

THE CONTROL OF FLUX THROUGH THE ARGININE PATHWAY IN NEUROSPORA CRASSA

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ABSTRACT

The experiments described in this thesis were designed towards reaching an understanding of the control of flux through the arginine pathway in Neurospora crassa. The previous algebraic analysis of steady state enzyme networks has shown that the flux is a systemic property and that, typically, the relation between flux and effector is non-linear. It was therefore necessary in the present experimental analysis to apply the method of modulation, i.e. to determine the flux response to a series of small changes in the concentration of a putative effector. It was already known that arginine affects the rate of each step in the arginine pathway by controlling the amount (induction or repression) and/or activity (activation or inhibition) of the individual enzymes. The principal and largely successful aim of this study, therefore, was to develop and apply an experimental method whereby the mycelial arginine concentration could be predictably modulated over a wide range, under steady state conditions; then to monitor and interpret the systemic response, as indicated by the changes in i) the levels of other pathway intermediates, ii) the specific activities of the connecting enzymes and iii) the measured fluxes. This was achieved by controlling the rate of citrulline uptake and, consequently, the steady state concentration of mycelial arginine in the auxotrophic mutant arg-12. Ornithine then becomes an "end product" with its accumulated steady state concentration indicating the pathway flux. From the experimentally generated relationships between metabolic pools and between pools and enzyme specific activities the mechanisms of importance in the control of the pathway fluxes were revealed. It is shown that there is no unitary solution to the problem of flux control; a number of different mechanisms effect the control of flux through the arginine pathway with the proportion of the control exerted by each depending absolutely upon the level that the flux has.

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1. INTRODUCTION

1.1 The objectives of the present study: immediate background

Experiments described in this thesis were carried out to elucidate further ^{the} factors regulating arginine biosynthesis and degradation in the Ascomycete fungus Neurospora crassa. Earlier investigations from this laboratory demonstrated the effect of quantitative variation in enzyme specific activity on the metabolic flux through the pathway. These variations in enzyme activity were achieved by using (a) geographically-isolated and genetically-distinct wild-type populations (Curtis, 1965), (b) heterokaryons containing different doses of mutant and normal alleles (Watson, 1969; Tateson, 1971) and (c) partial revertants of mutant alleles (Donachie, 1961; Barthelmess et al., 1974). A general theory of the control of flux through metabolic pathways has also been developed (Kacser and Burns, 1973 and 1979) and it is gratifying, though not surprising, that the results from the aforementioned individual studies are in complete accord with the predictions of the generalised theory. Flint (1977), in this laboratory, examined the role of compartment¹ of pathway intermediates in the regulation of flux to and from arginine.

The particular aim of the work described in this thesis has been to develop an experimental system by which flux through the same arginine pathway could be controlled "externally" (rather than "internally" by varying the activities of enzymes mutationally or as a consequence of variations in gene dosage), to study the consequences of such externally imposed conditions on intracellular fluxes, and to deduce

¹ The literature on this subject has made use of the terms compartmentalise, compartmentalised and compartmentalisation or compartmentation. Following Rolleston (1972) the least redundant and most precise and succinct verb, adverb and noun namely compartment, compartmented and compartmentation, have been used throughout this thesis.

the nature of the intracellular regulatory mechanisms that must have been operating during the experiments. The work to be described is thus a natural extension of earlier work from this laboratory. The impact of work from this and other laboratories to the present investigation will be discussed later in the thesis. The bulk of this Introduction will be devoted to descriptions of the characteristics of Neurospora crassa, of the arginine metabolic pathway and of current approaches to the understanding of the control of metabolic flux.

1.2 Characteristics of Neurospora crassa

Neurospora crassa occupies a distinguished position as an experimental organism for genetic research. The contribution of experimentation with Neurospora crassa to the field of genetics has been extensively documented (see Fincham and Day, 1971 and Whitehouse, 1973). The characteristics of this organism which favoured its use in the past, were essential to the type of system analysis proposed and are important to an understanding of the experimental approach adopted will therefore be only briefly outlined.

Neurospora crassa is a heterothallic fungus belonging to the Ascomycete class. The principal feature of the class is the ascus; formed in the perithecium (the sexual fruiting body), the asci contain the meiotic products, ascospores. In Neurospora crassa fertilization only occurs between strains of opposite mating type, A and a. After cell contact and membrane fusion between two such strains, nuclear migration and, subsequently, nuclear fusion takes place to form a diploid zygote within the ascus. Two meiotic divisions take place to produce eight, linearly arranged, haploid ascospores. After maturation the ascospores are expelled from the ascus and, under suitable conditions, they germinate to produce a rapidly growing, branched multi-nucleate mycelium. The haploid vegetative cycle is maintained by the formation of conidia which, on germination, produce fresh branching hyphae.

The principal advantages of this organism for genetic and biochemical studies may be summarised.

(a) Vegetative growth is rapid and exponential with a doubling time of approximately three hours. Neurospora crassa grows on fully defined liquid (or solid) medium which, in the case of wild-type, can

consist solely of a simple carbon source (e.g. glucose, sucrose or glycerol), biotin and inorganic salts. The requirements of auxotrophic mutants can be relieved by the translocation of an exogenously supplied compound which is subsequent, metabolically, to the enzyme deficiency. These characteristics are particularly relevant to the work to be described. In addition, the growth morphology of Neurospora is an important advantage to the type of study to be described. A consequence of the multinucleate, syncytial nature of the fungus in vegetative growth is that this organism is effectively in one metabolic phase at any one time; there are no cells as such and therefore none of the problems inherent in the analysis of multicellular tissues where more than one cell type may be present each in a different metabolic state nor, as with unicellular organisms, the problems associated with specific metabolic activities being discontinuous within the cell cycle.

(b) The sexual cycle is short (approximately ten days under optimum conditions) and controlled genetic crosses can be constructed. The relative ease of phenotypic and genotypic analysis of meiotic products coupled with their linear arrangement within the asci has, for example, lead to considerable insight into the phenomenon of gene conversion and the mechanism of genetic recombination (Whitehouse, 1973).

(c) Heterokaryons are readily formed between compatible strains through mycelial (but not nuclear) fusion (Beadle and Coonradt, 1944). Heterokaryosis is a powerful test for establishing functional allelism (or otherwise) of phenotypically identical mutants and for investigating the hetero- or homo-multimeric nature of proteins. As

alluded to in section 1.1 the ability, through heterokaryosis, to control gene dosage and hence study the effects of variation in enzyme activity can lead to insight (Watson, 1969; Tateson, 1971 and Kacser and Burns, 1973).

(d) Mutations can be easily induced, selected, isolated and characterised by standard genetic methods (Fincham and Day, 1971).

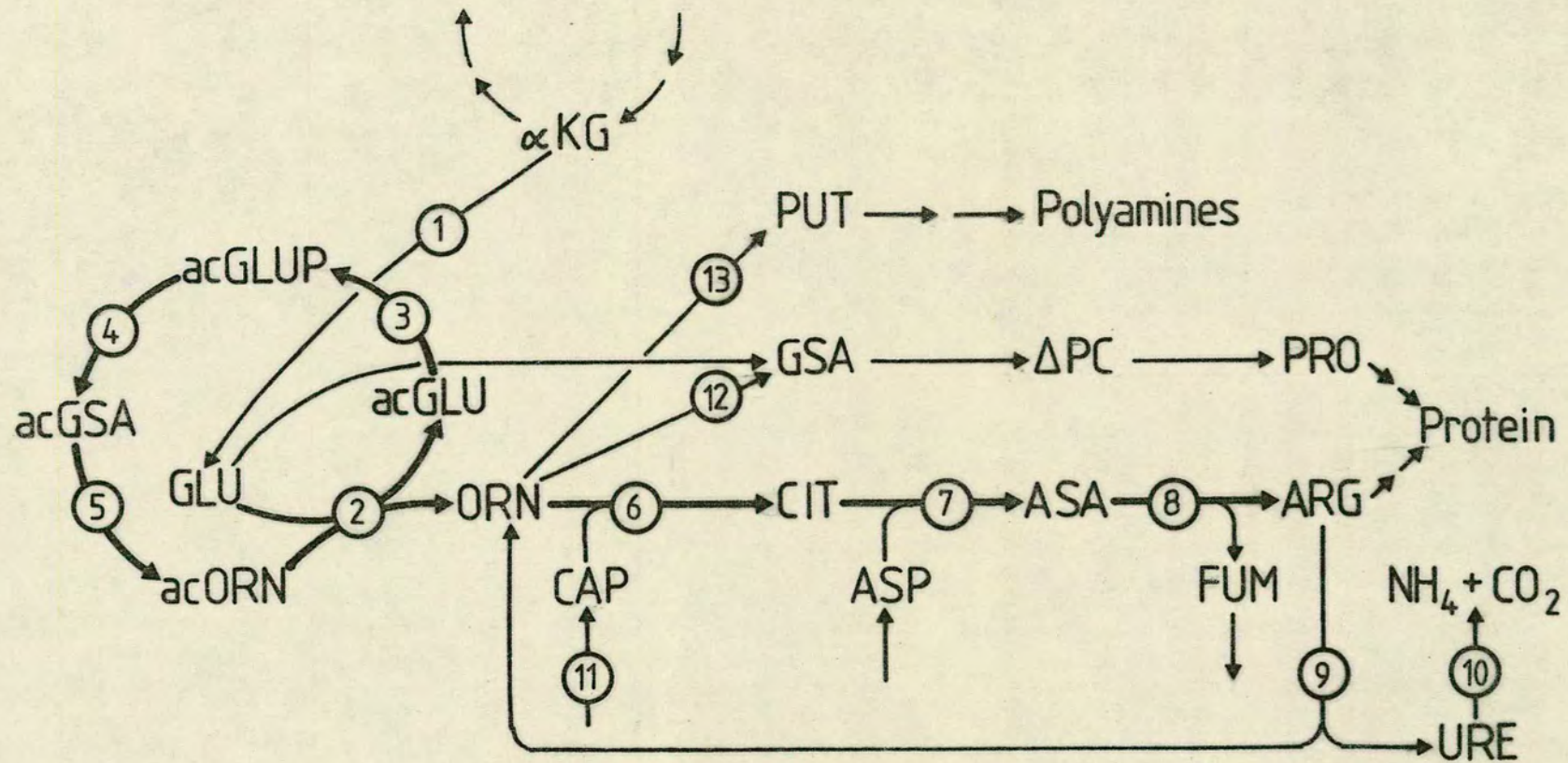
The experimental advantages conferred by the biology of this fungus were recognised by Beadle and Tatum (1941) in their endeavour to determine how genes control known biochemical reactions; Neurospora crassa became the first organism in which auxotrophic mutants were induced and their metabolic consequences characterised (Beadle and Tatum, 1941). (The idea of a connection between genes and enzymes had been present for nearly forty years previous, almost from the time of the rediscovery of Mendel's theory of inheritance. Early attempts to establish the link sought to do so by examining the biochemical nature of known genetic characters (e.g. Garrod, 1909). The evidence prior to Beadle and Tatum (1941) for a direct relationship between gene and enzyme was substantial but not conclusive). The methodology of Beadle and Tatum (1941) has been widely used; their experimental procedure will be briefly outlined.

Conidia were X-irradiated, crossed with a strain of opposite mating-type and the resulting ascospores germinated and cultured on "complete" medium. Three mutants were found which grew normally on "complete" medium but hardly at all on "minimal" medium. The nature of the induced mutations was established by systematically adding particular compounds to "minimal" medium to establish what, if any, synthetic capacity was deficient. By this method Beadle and Tatum (1941) established that the three mutants were associated with the

inability to synthesize pyridoxine, thiamin and para-aminobenzoic acid respectively. They showed, by genetic analysis, that each of these character differences differed from wild-type by a single unit of inheritance. The "one gene - one enzyme" hypothesis based on their work forged the link between Mendelism and biochemistry and catalysed the pursuit for an understanding of the underlying relationship between the genetic constitution and metabolic capacity of organisms. (It should be realised that the type of mutations originally isolated and characterised by Beadle and Tatum (1941) was fortuitous. Had they induced mutations affecting heteropolymeric, multimeric or "regulatory" enzymes the illuminating but, in retrospect, oversimplified "one gene - one enzyme" hypothesis might not have been formulated. The rapid advance which we have witnessed in our understanding of the gene-enzyme relationship and enzyme structure and function would probably have been seriously retarded).

Ever since the pioneer work of Beadle and Tatum (1941) Neurospora crassa has been a frequent choice for scientific investigation (Fincham and Day, 1971; Whitehouse, 1973). Consequently we now have an extensive knowledge and understanding of the genetics, biochemistry and physiology of this organism on which to base further studies.

Figure 1: The arginine pathway in Neurospora crassa.



Legend to Figure 1:

Metabolites; abbreviations:

α KG, α ketoglutarate; GLU, L-glutamate; acGLU, N-acetylglutamate; acGLUP, N-acetylglutamate phosphate; acGSA, N-acetylglutamate semialdehyde; acORN, N-acetylornithine; ORN, L-ornithine; CIT, L-citrulline; ASA, argininosuccinate; ARG, L-arginine; URE, urea; CAP, carbamoyl phosphate; ASP, aspartate; FUM, fumarate; GSA, glutamate semialdehyde; PC, Δ -pyrroline-5-carboxylate; PRO, L-proline; PUT, putrescine.

Enzymes:

Step	Genetic Locus	Abbreviation	Enzyme
1	am	GDHase	NADP-glutamate dehydrogenase
2	arg-4,7	AOGTase	acetylornithine glutamate transacetylase
3		AGKase	acetylglutamate kinase
4	arg-6	AGPreductase	acetylglutamate phosphate reductase
5	arg-5	AOTase	acetylornithine transaminase
6	arg-12	OTCase	ornithine transcarbamylase
7	arg-1	SYNase	argininosuccinic acid synthetase
8	arg-10	ASAase	argininosuccinase
9	aga		arginase
10	ure		urease
11	arg-2, arg-3	CPSase-ARG	carbamoyl phosphate synthetase-arginine pathway specific
12	ota	OTAase	ornithine transaminase
13		ODCase	ornithine decarboxylase

Legend to Figure 2:

Metabolites; abbreviations:

HIS, L-histidine; ASP, aspartate; PYR, pyrimidines.

All other abbreviations, see legend to Figure 1.

1.3 The arginine metabolic pathway in *Neurospora crassa*

A knowledge of the metabolic structure, sub-cellular organisation and regulation of the arginine pathway in *Neurospora crassa* is prerequisite to an understanding of the experimental strategy adopted in this work, of the results obtained and their interpretation. Two flow diagrams illustrate the essential features of arginine synthesis and degradation. Figure 1 shows the metabolic routes to and from arginine and the metabolic relationship of the arginine pathway to the biosynthesis of proline. The enzymes which catalyse individual reactions in these metabolic routes and the structural genes specifying the enzymes are given in the accompanying legend. Figure 2 displays the same metabolic pathways (together with those leading to pyrimidine synthesis), depicts the compartmenting of the intracellular components and indicates some of the relationships between the intracellular and extracellular environments. It should be appreciated that the structural elements and transformations illustrated in Figure 2, additional to those described in Figure 1, while identifying a hierarchy of biological organization, are simply and formally equivalent to an extension to the metabolic structure. The translocation of CIT from the medium to the cytosol or of ARG from the cytosol to the vesicle are no less transformations than the conversion of CIT to ARG via ASA. Each transformation, whether locational or chemical, is a kinetic process; the concentrations of pre- and post-transformed components are in thermodynamic relations whether as "substrate" and "product" of an enzymic chemical transformation or as "X EXTERNAL" and "X INTERNAL" of a (enzyme mediated?) locational transformation.

With regards to this thesis in particular, the following features of the arginine metabolic pathway in Neurospora are important.

(a) We can be confident that all substrates, cofactors, intermediates and end-products of the arginine metabolic pathway are known; so too are the enzymes which catalyse specific steps in this pathway. We are equally well informed about those related pathways which compete with arginine for carbon- and nitrogen- containing precursors. We are less well informed about the mechanisms facilitating translocation of substrates across extra- and inter-mycelial membranes though the phenomenon of translocation of specific solutes is well documented and will be dealt with in more detail presently.

(b) Methods are available for determining the concentration of each of the metabolites shown and enzymes listed in Figures 1 and 2. Methods are also available for determining rates of translocation of solutes into and out of membrane-enclosed compartments. A particular and important advantage of working on the arginine pathway is that most of the metabolites are Ninhydrin-positive and separable by an aminoacid autoanalyser and therefore each metabolite concentration can be determined in a single culture sample with a single analytical procedure.

(c) Methods are available for selecting mutants in which one or more enzymes or membrane-translocation mechanisms is partially or fully defective.

(d) The subcellular organisation of the pathway (see Figure 2) may be assumed to play a significant role in the regulation of arginine biosynthesis and degradation. Equally significant is the

regulation of enzyme activity by repression, induction and/or inhibition mechanisms.

How these features were established will be described.

1.3.1

The metabolic structure of the pathway

Srb and Horowitz (1944) investigated the steps involved in the synthesis of L-arginine making use of the method of Beadle and Tatum (1941) (see 1.2) for the biochemical characterisation of induced mutations. They isolated fifteen mutants, induced by either X- or UV-irradiation, which were found to map at seven different loci each leading to an inability to synthesize L-arginine. They confirmed the functional allelism of mutants mapping at the same locus by the heterokaryosis method of Beadle and Conrardt (1944). The seven genetic classes of mutant were divisible into three phenotypic classes on the basis of their nutritional requirements; four genetic classes were found to grow on minimal medium supplemented with L-ornithine, L-citrulline or L-arginine, two classes only when supplemented with L-citrulline or L-arginine and one class only when supplemented with L-arginine. They concluded that L-arginine was synthesised in Neurospora crassa by a mechanism involving at least seven different genes and presumably seven different enzymes and resulting in the conversion of L-ornithine to L-citrulline, the latter being the immediate precursor of L-arginine. Arginase and urease enzyme activities were also detected in wild-type Neurospora crassa suggesting that an ornithine cycle, similar to the urea cycle previously demonstrated in mammals, might operate.

Since the pioneer work of Srb and Horowitz (1944), the complete arginine metabolic pathway has been characterised biochemically and genetically (see Figure 1 and accompanying legend). This history will be outlined.

Newmeyer (1957 and 1962) demonstrated that the class of mutants showing growth only on L-arginine supplemented medium was composed of mutations mapping at one or other of two non-allelic loci, designated arg-1 and arg-10 respectively. All arg-1 mutants lacked argininosuccinic acid synthetase (SYNase) activity and accumulated L-citrulline (CIT) in the mycelium. Fincham and Boylen (1957) demonstrated that all mutants at the arg-10 locus lacked argininosuccinase (ASAase) activity and accumulated argininosuccinate (ASA) in the mycelium. Donachie (1961) confirmed arg-10 as the structural locus for ASAase by the demonstration of altered Km's and pH optima for the enzymes formed by arg-10 revertants.

A mutant, originally designated s and described as a suppressor of pyrimidine-requirement by Houlahan and Mitchell (1947), was shown by Davis (1962a and b) to have an approximately 97% reduction in ornithine transcarbamylase (OTCase) activity with respect to wild-type. The OTCase enzyme formed in the mutant also had altered Km's for both of its substrates, carbamyl phosphate (CAP) and L-ornithine (ORN). Mutants completely deficient in OTCase activity were selected from s and also directly from wild-type (Davis and Thwaites, 1963). All mutations were found to be allelic and the structural gene locus for OTCase was designated arg-12 with s being redesignated arg-12^s.

Davis (1963) described two other classes of mutant which behaved nutritionally in the same way as arg-12 mutants but which had normal OTCase activity. These two classes of non-allelic mutants, designated arg-2 and arg-3, were both found to lack carbamyl phosphate synthetase activity (Davis, 1965a and b and 1966). Davis (1965b) proposed that the two loci coded for different sub-units of a heteromultimeric protein which catalysed the formation of carbamyl phosphate (CAP) from glutamine (GLN) and CO_2 .

Vogel and Bonner (1954) described mutants (later designated arg-8 and arg-9) capable of growth on L-arginine, L-proline, L-citrulline, L-ornithine or glutamic semialdehyde. They proposed that glutamic semialdehyde was a common precursor of both arginine and proline. Later evidence suggested however that the principle source of L-ornithine was via acetylated intermediates. Vogel and Vogel (1963) detected acetylornithine transaminase (AOTase) activity in mycelial extracts and acetyloronithine glutamate transacetylase (AOGTase) activity found to be absent in arg-4,7 mutants. Morgan (1965) demonstrated that a class of ornithine requiring mutants, designated arg-5, accumulated acetylglutamic semialdehyde and were deficient in AOTase activity.

L-arginine is hydrolysed to urea and L-ornithine by arginase. This activity is absent in aga strains (Davis et al , 1970 and Morgan, 1970) which are characterised by the inhibition of growth on arginine and supplemented medium. This growth inhibition is reversed by the addition of L-ornithine, spermidine or putrescine to the growth medium (Davis et al , 1970). Putrescine, which is

formed by the decarboxylation of L-ornithine (ODCase activity), is essential for growth. The growth inhibition by L-arginine of aga strains can be understood in terms of feedback inhibition by arginine of ornithine biosynthesis (see 1.3.2.2) coupled with the lack of catabolically-derived ornithine normally produced by arginase activity (Davis et al, 1970).

L-ornithine is converted to glutamic semialdehyde by ornithine transaminase (OTAase). This activity is absent in ota strains (Davis and Mora, 1968). Although imposing no requirement in itself, the effects of the OTAase-less mutation when in combination with certain arg mutants is of interest to an understanding of the regulation of arginine metabolism to be discussed in the next section.

Urea is hydrolysed to CO_2 and NH_3 by urease, the activity of which is dependent on the product of two non-allelic loci, ure-1 and ure-2 (Kölmark, 1969a and b).

1.3.2. The organisation and regulation of the pathway

The main purpose of this thesis has been directed towards an understanding of the control of flux through the arginine pathway. Two general phenomena are illustrated by the pathway and are intuitively of relevance to control of flux. These will be described.

1.3.2.1 Compartition of enzymes and intermediates

Before proceeding with this section it is pertinent that a clear distinction is made between what is meant by phenomena and what is meant by mechanisms. In so doing certain descriptive terms which will be used repeatedly in the text will be defined. Inspection of

the metabolic map reveals that several metabolites are common to more than one pathway, being produced and/or utilised by different enzymes. A frequent observation is that such metabolites, however produced, are not equally available for utilization by the sharing pathways. This phenomenon, that the metabolic fate of a common intermediate is to an extent determined or restricted is generally referred to as channeling. A pool of an intermediate normally destined to a particular metabolic fate may, under certain circumstances, become available to another utilising pathway. This phenomenon is generally referred to as de-channeling or, alternatively, the pool is said to spill-over to other pathway. The terms channeling, de-channeling and spill-over describe experimental observations; no mechanism underlying these phenomena is implied by the use of these terms.

Mechanisms can be defined as demonstrable structural components of a system capable, at least potentially, of determining a particular phenotypic character or, more particularly, of resulting in an observed phenomenon. The sub-cellular localisation, compartmentation and sequestration of enzymes and intermediates are mechanisms concluded to effect the phenomenon of metabolic channeling specifically and to be of importance to the regulation of metabolism in general. Localisation is used specifically to mean a restriction or concentration of components to or in a particular sub-cellular fraction. Compartmentation similarly implies a heterogenous sub-cellular distribution but specifically means a division of the total amount of a component between more than one sub-cellular compartment separated from one another physically and/or kinetically. Finally, sequestration denotes the seclusion, isolation or setting-apart either kinetically or

physically of a component. Thus, the compartmentation of the ornithine pool, more than 90% of the total extractable being sequestered within the vesicle, together with localisation of producing and utilising enzymes has a significant effect in determining the fate of biosynthetically derived, as opposed to endogenously supplied or catabolically derived, ornithine.

(a) Carbamyl phosphate

Carbamyl phosphate (CAP) is an intermediate of both the arginine and the pyrimidine metabolic pathway (Figure 2). The evidence for the channeling of CAP and the mechanisms whereby it is achieved have been reviewed (Davis, 1967 and Reissig et al., 1967); only the essential elements will be described here.

(i) Unlike E.coli, Neurospora crassa has two carbamyl phosphate synthetase enzyme activities, one specific to the pyrimidine pathway (CPSase-PYR), the other specific to the arginine pathway (CPSase-ARG). These activities are absent in pyr3a and in arg-2 and/or arg-3 mutants respectively. The arg-2 and arg-3 loci code for the two different subunits of a heteromultimer which has CPSase-ARG activity (Davis, 1965a and b and 1967).

(ii) CAP is utilised by the pyrimidine pathway enzyme aspartate transcarbamylase (ATCase) and by the arginine pathway enzyme ornithine transcarbamylase (OTCase). These enzyme activities are absent in pyr3d and in arg-12 mutants respectively (Reissig et al., 1965 and Thwaites, 1963).

(iii) The pyr-3 locus (including both pyr3a and pyr3d mutations) specifies a bifunctional protein having both CPSase-PYR and ATCase activity (Davis, 1967; Williams and Davis, 1970 and Williams et al., 1970 and 1971). These activities are largely restricted to the

nucleus (Bernhardt and Davis, 1972).

(iv) The location and activity of CPSase-ARG and OTCase are restricted to the mitochondria (Bernhardt and Davis, 1972 and Weiss and Davis, 1972).

(v) The metabolic fate of CAP is assured by the sequestration of the synthetase and transcarbamylase of the arginine pathway from those of the pyrimidine pathway by the nuclear and mitochondrial membranes. This physical mechanism for the channeling of CAP is enhanced by a kinetic mechanism; CPSase-PYR and ATCase activity are functions of the same protein and consequently most of the pyrimidine pathway CAP is not free but present in an enzyme-substrate form (Williams et al, 1970 and 1971).

(vi) The suppressive effect of the arg-12^S mutation on the pyr-3a mutation is explained as follows. The arg-12^S mutation results in the reduction of OTCase activity to about 3% (Davis, 1962a and b) and, as a consequence, a reduction in the arginine pool to about 10% of that observed in wild-type grown on minimal medium (Barthelmess et al, 1974). As a consequence of the lowered arginine pool anabolic enzymes of the arginine pathway, including CPSase-ARG, are derepressed (Cybis and Davis, 1975). The net result of a decreased OTCase activity and an increased CPSase-ARG activity is that CAP accumulates and "spills-over" to the pyrimidine pathway, thus satisfying the CAP-requirement imposed by the pyr-3a mutation; (Davis, 1967; Reissig et al, 1967). The suppressive effect of the arg-12^S mutation is annulled when pyr3a arg-12^S double mutants are grown on arginine supplemented medium; the resultant high internal arginine pool causes the repression of CPSase-ARG activity (Cybis and Davis, 1975) and hence

there is no accumulation or "spill-over" of CAP. In a reciprocal way, the suppressive effect of partial revertants of pyr3d mutations on arg-2 or arg-3 mutations is explained by CAP, normally destined solely to the pyrimidine pathway, accumulating and "spilling-over" to satisfy the requirement for CAP by the arginine pathway (Davis, 1967 and Reissig *et al.*, 1967). The channeling of CAP is a good illustration of the regulatory significance of compartmentation. Of more direct relevance to this work is the observation and implications of the compartmentation of the ornithine and the arginine mycelial pools.

(b) Ornithine and Arginine

The growth requirements of the arg-8,9 mutants (see 1.3.1) provided the first evidence that mycelial ornithine exists in more than one pool with different metabolic sources and fates. Vogel and Kopac (1959) demonstrated that the basic requirement of these mutants was for L-proline (PRO); L-ornithine (ORN) supported growth through its conversion to glutamic semialdehyde (GSA) and thence to PRO, L-arginine (ARG) (and also L-citrulline (CIT) through catabolism firstly to ORN and then to GSA (see Figure 1 and Legend). The inability of arg-8,9 mutants to grow on minimal medium was interpreted (Davis, 1968 and Davis and Mora, 1968) as evidence for biosynthetically and exogenously derived ORN being sequestered and having different metabolic fates, the former being principally destined to an anabolic fate with the latter being substantially available for catabolism to GSA.

Further evidence for multiple ornithine pools came from the growth requirements of arg-5 and arg-12^S single mutants and the arg-5 arg-12^S double mutants (see Figure 1 and accompanying legend

and 1.3.1). It was found that the concentration of ORN in the medium required to support the growth of the arg-5 arg-12^S double mutant was 100-fold higher than that required by the arg-5 single mutant (Davis, 1968). This observation had to be reconciled with the fact that the arg-12^S mutation itself does not impose a nutritional requirement (Houlahan and Mitchell, 1947). It was suggested by Davis (1968) that the mutationally-altered OTCase enzyme, determined by arg-12^S, is able to compete successfully with OTAase for biosynthetically derived ORN but, unlike normal OTCase, cannot compete successfully with OTAase for exogenously supplied ORN. This view was substantiated by the finding that strains can be selected from arg-5 arg-12^S double mutants which are able to grow on low concentrations of ornithine in the medium (Davis and Mora, 1968). This ability is dependent on an additional enzyme deficiency, namely OTAase (Davis and Mora, 1968); in the absence of a competitive catabolic fate the mutant OTCase is able to utilise exogenously derived ORN (Davis and Mora, 1968).

The prototrophy of arg-8,9 arg-12^S double mutants is presumed to result from the elevated biosynthetically derived ORN pool (a consequence of the arg-12^S mutation) being sufficient to allow catabolism by OTAase to GSA and hence relief of the PRO requirement imposed by the arg-8,9 mutation (Davis, 1968). This inference is supported by the observation that arg-8,9 arg-12^S ota triple mutants are obligatory PRO requirers (Davis, 1968). The slight leakiness of arg-8,9 mutants is probably due to a very low level of ORN catabolism to GSA occurring in cultures grown on minimal medium.

These observations of the single and combined effects of complete and partial enzyme deficiencies support the thesis that (a)

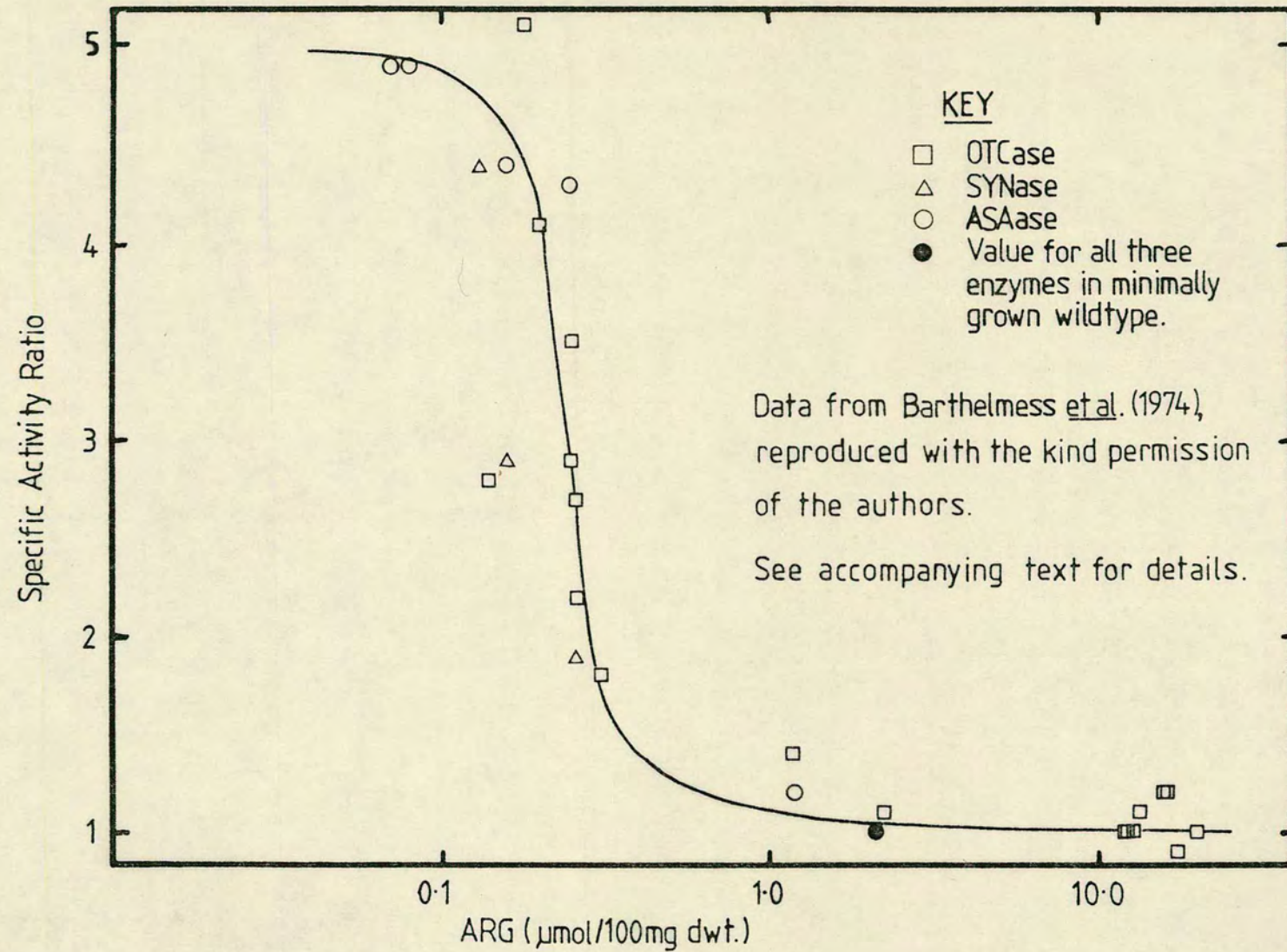
biosynthetically derived and exogenously derived ORN are sequestered within the mycelium and that (b) biosynthetic ORN has a predominantly anabolic fate being preferentially available to OTCase whereas exogenously derived ORN has a predominantly catabolic fate being preferentially available to OTAase.

It is now established that ORN is located in three different sub-cellular compartments and while all of these pools equilibrate with one another they each have predominantly different metabolic fates (Weiss, 1976; Karlin et al, 1976; Bowman and Davis, 1977a and b).

Weiss (1973) demonstrated by cell fractionation studies that more than 90% of the ORN and ARG in Neurospora mycelium is associated with an osmotically fragile, probably membrane bound, subcellular organelle. This organelle, called the "vesicle", is distinct from the mitochondria, glyoxysomes and vacuoles (Weiss, 1973). The results of pulse labelling (Subramanian et al, 1973; Karlin et al, 1976; Weiss, 1976 and Bowman and Davis, 1977a and b) and label equilibrium (Flint, 1977; Karlin et al, 1976 and Bowman and Davis, 1977a and b) studies suggest that the physical compartmentation of the ornithine and arginine pools is an effective mechanism for the metabolic channeling of these aminoacids. The following general conclusions can be drawn from these studies.

- (i) More than 90% of the total mycelial ORN and ARG is sequestered in a metabolically inactive compartment, the vesicle.
- (ii) The ARG not held in the vesicle is located in the cytosol.
- (iii) Less than 5% of the total mycelial ORN is located in the mitochondria and about 1% is cytosolic.
- (iv) The aminoacid pools in different sub-cellular compartments

Figure 3: The Bradytrophic Derepression Curve.



equilibrate with one another although each pool has a predominantly different metabolic fate.

The effect of the compartmentation of metabolic pools on the regulation of the arginine pathway will become apparent when the evidence for the subcellular localisation of the pathway enzymes is considered.

(c) The subcellular localisation of arginine pathway enzymes.

The following subcellular locations of the arginine pathway enzymes have been established by the co-purification of enzymes with marker enzymes (Weiss and Davis, 1973), histochemical techniques (Bernhardt and Davis, 1972) and cell fractionation studies (Weiss and Davis, 1973 and Cybis and Davis, 1975).

(i) AGKase is a cytosolic enzyme; all of the other enzymes of the acetyl cycle are mitochondrial. (Cybis and Davis, 1975; Weiss and Davis, 1973).

(ii) The location of both CPSase-ARG and OTCase is restricted to the mitochondria (Bernhardt and Davis, 1972). The remaining anabolic enzymes SYNase and ASAase are cytosolic (Weiss and Davis, 1973; Cybis and Davis, 1975).

(iii) The catabolic enzymes arginase, urease and OTAase are all cytosolic (Weiss and Davis, 1973).

(iv) Arginyl-tRNA synthetase, the enzyme which charges arginyl-tRNA with ARG, and ODCase, the enzyme which decarboxylates ORN to PUT, are both cytosolic (Weiss and Davis, 1973).

Taking together the evidence for the subcellular localisation of enzymes and the compartmentation and sequestration of intermediates, Davis and colleagues have interpreted the data in the following way.

Exogenously supplied arginine or ornithine enters the cytosol where it has a predominantly catabolic fate (Davis, 1968; Weiss and Davis, 1977). A physical association with the outer cell membrane for the utilising enzymes arginase and OTAase has been postulated (Karlin et al, 1976 and Flint, 1977). ORN is formed and consumed biosynthetically within the mitochondria; very little enters the cytosol where it can be catabolised (Bowman and Davis, 1977a and b). The concentration of biosynthetically derived ORN in the cytosol is probably less than the K_m for OTAase (Weiss and Davis, 1973) which explains the observation that transemination of ORN to GSA in cultures grown on minimal media is insignificant (Davis, 1968). The affinity for ORN of ODCase is much higher; some of the cytosolic ORN can be used for polyamine biosynthesis (Weiss and Davis, 1973).

The majority of the ARG formed anabolically in the cytosol is sequestered to the vesicle (Weiss, 1973 and Subramanian, et al, 1973). The low cytosolic concentration of arginine, which is probably less than the K_m for arginase, restricts the amount of catabolism to ORN (Subramanian et al, 1973; Weiss and Davis, 1977). Localisation of enzymes and compartmentation of intermediates serves to effectively sequester exogenously derived from endogenously derived ornithine and arginine, to separate the anabolic and catabolic pathways and hence to limit the potentially "wasteful" cycling of ORN (Bowman and Davis, 1977a and b; Karlin et al, 1976).

While the terms and concepts in which the foregoing experimental interpretation are framed are open to criticism it is nevertheless clear that the compartmentation of enzymes and intermediates is an important regulatory feature of the arginine pathway in Neurospora

crassa. The second mechanism of significance to the control of flux through the pathway is the regulation of enzyme activity.

1.3.2.2. Regulation of enzyme activity

Induction, repression and/or inhibition mechanisms regulate the activity of each of the enzymes in the arginine pathway in Neurospora crassa.

Derepression of each of the anabolic enzymes is observable under conditions leading to a reduction in the mycelial arginine pool below that observed in wild-type grown on minimal medium (Barthelmess et al, 1974 and Cybis and Davis, 1975) (Figure 3). CPAase-ARG activity is almost completely repressed by growth on excess arginine; there is about a 40-fold change in activity between fully repressed and derepressed conditions (Cybis and Davis, 1975). All the other anabolic enzymes show much lower levels of derepression (less than 5-fold) and are not repressed below their wild-type minimal media levels, (Cybis and Davis, 1975; Barthelmess, et al, 1974). The signal for derepression is arginine and the co-repressor is believed to be the internal arginine pool or a directly related metabolic pool (Barthelmess et al, 1974).

The activity of the acetyl-cycle enzyme, AGKase is, in addition, inhibited by arginine in vitro (Cybis and Davis, 1974). Consideration of the determined K_i for arginine in vitro, the assumed cytosolic arginine concentration and the cytosolic localisation of AGKase enzyme suggests that feed-back inhibition is operative in vivo (Cybis and Davis, 1975) and is consistent with the belief that the main regulatory signal for the pathway is the cytosolic arginine pool

(Cybis and Davis, 1975 and Barthelmess et al, 1974).

The catabolic enzymes, arginase, urease and OTAase, are each induced by arginine, the end-product of the anabolic pathway (Davis et al, 1970; Castaneda et al, 1967; Canedo et al, 1967 and Weiss and Anterasian, 1977). About a five-fold increase in these activities is observed following the addition of arginine to wild-type cultures grown on minimal medium (Weiss and Davis, 1977 and Weiss and Anterasian, 1977). This increase in activity is due to new protein synthesis and not the unmasking of pre-formed enzyme (Weiss and Davis, 1977 and Weiss and Anterasian, 1977). Little catabolism takes place in cultures grown on minimal medium despite significant levels of enzyme activities (Tateson, 1971; Subramanian et al, 1973; Weiss and Davis, 1977; Weiss and Anterasian, 1977). Explanations for these observations have been in terms of enzyme localization and the compartmentation of ornithine and arginine. In addition to these explanations must be added the evidence for the inhibition of arginase activity by ORN and CIT in vitro and in vivo (Mora et al, 1972 and Flint, 1977).

1.3.3 The arginine pathway in Neurospora: a summary

The metabolic and enzymic components of the arginine pathway in Neurospora are known and quantifiable. Superimposed upon the metabolic structure of the pathway (Figure 1) is another level of biological organisation, namely the subcellular localisation of enzymes and intermediates, their compartmentation and sequestration (Figure 2). These latter structures may be viewed simply as an extension to the description of molecular transformations embodied in the metabolic

map. They must therefore be taken into account in any analysis of the control of metabolic flow through the arginine pathway since they influence the availability of specific substrates to specific enzymes and therefore the rates of respective reactions. In addition, the rate of individual reactions and consequently the overall metabolic flow through the pathway will be affected by the mechanisms of repression, induction and/or inhibition of enzyme activities.

1.4 Current approaches to the understanding of the control of flux through intracellular metabolic pathways

Some consideration of the parameters and variables thought or known to control the flux of material through a metabolic system will help to describe the complexity of the system and the variety of efforts that have been made to understand it. It will also help to define the particular approach used in the present investigation.

A cell may be viewed as a space separated from the environment by a boundary (the cell-surface membrane) which is selectively permeable to the uptake and egress of ions and molecules, the exceptions being gases and water which appear to pass through biological membranes with exceptional ease. Within the cell space is a set of enzyme-catalyzed molecular transformations which maintain the system as a whole in a state of non-equilibrium. A cell, or organism, is thus a highly organised entity (by utilising the free energy of nutrients it staves off, at least temporarily, its own entropic doom) which maintains its molecular components at concentrations such that the overall chemical reactions of the system are not at thermodynamic equilibrium; individual components of the overall chemical reactions may be at, or very close to, equilibrium. A consequence of this situation is that there is always a net flux of material into, through and out of the cell or organism. Life is characterised by flux, death by the lack of it. A further feature of flux through a living open system is that the intracellular components may increase or decrease in concentration with the passage of time, or they may be maintained in a steady state for a period of time (Kacser, 1963; Kacser and Burns, 1973 and 1979).

Inspection of any reasonably complete metabolic map illustrates the complex inter-relationships of different metabolic pathways. Nevertheless even a complete elucidation of the metabolic map would not tell us how a cell or organism behaves, i.e. the quantitative aspects of its components. The behaviour of a cell is determined by the interactions between its component parts; it is not a simple sum of single steps. The universal phenomena of dominance, pleiotropy and epistasis confirm this view. The structure, or phenotype, of the organism will thus be determined by these interactions and their consequences on the relative fluxes through different metabolic pathways catalysed by specific arrays of enzymes and consequently on the 'accumulations' of all its molecular species in their various locations.

To approach an understanding of this we must have as much information as possible on the metabolic structure discussed above as well as on the interactions affecting the activities of the enzymes in such structures. From these it may be possible to describe in quantitative terms the molecular amounts and their rates of transformation which constitute the phenotype.

If we are to study the control of metabolism we must therefore ask what factors control the rate of enzyme-catalysed molecular transformations. The catalytic activity of an enzyme in vivo is a function of (i) the genetically determined concentration of enzyme present which may be variable (induction, repression) or considered to be a parameter and (ii) the kinetic properties of the enzyme defined by the K_m 's and V_{max} 's, again genetically determined parameters.

The K_m 's and V_{max} 's can become variables if the enzyme in question is subject to activation or inhibition; an alteration in either kinetic characteristic is operationally equivalent to a variation in the amount of enzyme present (Kacser and Burns, 1973) though the mechanisms involved are quite distinct. The flux through a metabolic pathway will also be influenced by the subcellular organisation, where enzymes are located and intermediates produced and consumed. The effect of the compartmentation of enzymes and intermediates on the regulation of metabolism is well illustrated by the arginine pathway in Neurospora crassa and has already been discussed as has the regulation of the activity of the arginine pathway enzymes (see 1.3.2.1 and 1.3.2.2). The characteristics of the arginine pathway in Neurospora crassa confer several advantages on the experimenter (see preceding sections). We might hope that study of this lower eukaryote will lead to insight into the behaviour of higher organisms less amenable to genetic analysis. If the analysis of a particular system is to tell us anything other than how that specific system behaves then we must establish that the specific phenomena illustrated by our system are general properties of metabolic systems.

1.4.1 Genetic systems and regulatory phenomena

A phenomenon of considerable importance for the regulation of flux through a metabolic pathway is the induction and repression of the synthesis of enzymes and of specific translocators or "carriers" which subsequently function as porters for the movement of selected solutes through membranes. The elegant elucidation by

Jacob, Monod and co-workers (see Jacob and Monod, 1961) of the lac operon system for the regulation of lactose utilization in E.coli K12 precipitated the search for similar genetic units of regulation of protein synthesis associated with other metabolic pathways in this and other organisms. The general and particular features of the operon system of controlling the synthesis of particular proteins are now too well known to require detailed description here. In contrast the search for operon systems in eukaryotes has met with little success; several fungal systems have been described where metabolically related enzyme activities are apparently coded by distinct but tightly linked loci which are expressed in a coordinate fashion but in most cases the evidence is insufficient to define an operon and alternative explanations for the observations are possible. Some of these genetic systems and the regulatory phenomena illustrated by them which are of greatest interest to the author and relevance to the present work are described in Appendix I to this thesis. Nevertheless, it is pertinent that some general observations and conclusions drawn from these studies are mentioned at this point.

(i) Although in its strictest form the operon system may be entirely restricted to the prokaryotes several elements of the system are illustrated by fungal (i.e. eukaryotic) systems in the control of enzyme activity by induction and repression mechanisms.

(ii) The regulation of enzyme activity through the control of the synthetic rate or by activation/inhibition mechanisms is by no means the only factor in any possible metabolic control; the compartmentation and sequestration of enzymes and intermediates, a widespread observation in eukaryotic cells, is an equally efficacious

mechanism for the control of metabolic flow. The highly complex, membrane bound, subcellular organisation of even the simplest eukaryote distinguishes it from the prokaryotic cell type. The strategy for metabolic control by compartmentation, sequestration et alia, can be seen as the direct product of the heterogenous nature of the eukaryotic cell and it follows that it is largely excluded as a strategy which the relatively homogeneous prokaryotic cell can exploit.

(It may be concluded, teleologically, that the 'operon' system is ideally suited to the prokaryotic cell. They have a short life cycle and unable to store essential metabolites to any great extent. They must therefore be able to respond rapidly to the varying concentrations of nutrients in their surrounding, natural environment with large changes in the synthetic rate of relevant enzymes. By the same philosophy, the "operon" system is not the optimum strategy for eukaryotes with their longer generation times and their ability to store, intracellularly essential metabolites to various extents. In general, the degrees of induction and repression of enzyme activity in eukaryotes ^{are} higher in unicellular organisms, where relatively large and continual changes in the normal external environment might be expected, compared with multicellular organisms, where the organism is buffered internally to a large extent against changes in the external environment. In higher organisms only a few specialised cells are exposed to the fluctuating external environment with the majority existing in a self generated, homeostatic environment. In these organisms large changes in enzyme synthetic rates are only seen in normal development in association with dramatic changes in the developmental or differentiated state).

(iii) Closely related organisms may demonstrate different mechanisms for solving the same problem of metabolic control although the general similarities in achieving the overall solution are striking.

(iv) The sphere of influence of genetic control systems is not restricted to individual pathways; hierarchies and complexities of control permeate and integrate the whole of metabolism.

1.4.2 Kinetic and energetic considerations; the determination of enzyme activities and metabolite concentrations.

In the preceding sections (1.3 and 1.4.1) and in Appendix I genetic systems and regulatory phenomena have been described which we believe, at least intuitively, are involved in the control of flux. From a consideration of these mechanisms certain inferences as to their effects in vivo can be and have been made, e.g. the system's response to the presence or absence of an exogenous supply of a metabolite in the level of its catabolism and endogenous biosynthesis. It is, however, a characteristic of such an approach that, at best, qualitative conclusions can be reached applying particularly to "extreme" conditions. Such conditions, while they may reveal the existence of a mechanism (or are shown to be consistent with it) hardly ever cover the range of "normal" variation. Yet it is this range which is at least an equally worthy object of investigation. In fact, most of the work reported so far is in terms of structure and mechanism. The "synthesis" of all this information has rarely been attempted and, as will be shown later, requires a different methodology and concepts than are usual in this field.

Workers in this field are not unaware of the need for applying the 'in vitro' information to in vivo situations. The approaches which have been made in this direction will be discussed.

Measurement of enzyme activities, of K_m and V_{max} on isolated enzymes in vitro is technically relatively easy. Rapid accumulation of data of this kind has naturally lead to attempts to identify "pacemaker", or "rate-limiting" or rate-controlling" enzymes amongst the array of enzymes in particular metabolic pathways. As will be shown, the deductions from such measurements can be, at best, intuitively plausible. The measurements themselves are nevertheless valuable. Rolleston (1972) has given an extensive treatment of the three main approaches to an understanding of control of flux that fall under this section; the following is an abbreviated account.

1.4.2.1 The teleological approach

This approach depends on the premise that "control sites" (i.e. "rate-limiting enzymes") should occur early in a metabolic pathway and/or shortly after branch points in a linear pathway; the contention is that such an organisation would prevent long stretches of "uncontrolled" metabolic flux and would be more "economical". The approach requires familiarity with detailed metabolic maps but no experimentation and is thus untestable.

1.4.2.2 Analysis of the properties of isolated enzymes

The premise of this approach is that enzymes with relatively low maximum activities or which exhibit allosteric properties (i.e. which are subject to reversible activation or inhibition by intra-

cellular metabolites) are likely to be "regulatory" or potentially "rate-limiting" enzymes. This approach requires extensive analysis of the properties of individual enzymes in vitro. That an enzyme exhibits properties consistent with a role as "controlling" enzyme does not, however, permit the conclusion that it does in fact play this role in vivo since there is no necessary relationship between the behaviour of an enzyme in vivo and in vitro. This is not only because the properties of an enzyme preparation in vitro can almost never be studied under physiological conditions. Principally it is because no enzyme in vivo is active in isolation; the flux through a metabolic pathway is a systemic property which depends upon all components of the pathway (Kacser and Burns, 1973). Furthermore, there are purely technical reasons why in vitro measurements may be misleading, for example, the total extractable concentrations of enzymes and metabolites often bear little relation to the concentrations "seen" in vivo because of two widespread mechanisms - compartmentation of enzymes and sequestration of substrates. The eukaryotic cell has a complex subcellular organisation which affects the regulation of biosynthetic pathways through the compartmentation of enzymes and metabolites (Davis, 1975). Sols and Marco (1970) have discussed the metabolic consequences of the subcellular microenvironment and compartmentation, particularly for the case where metabolite concentrations are low. They conclude that, as a consequence of compartmentation, enzyme concentrations may be of the same order as, if not higher than, that of its substrate(s). It follows that (depending, of course, on what the relevant K_m 's are)

such enzymes may rarely be saturated with substrate in vivo and thus the reaction rate will be substrate concentration dependent. Fallacious conclusions may be formed if the enzyme is assumed to "see" the total extractable concentration of substrate; it is the concentration of free substrate that is required in considering the kinetics and the energetics of flux through metabolic pathways. Not only will compartmentation have a potential effect on the free substrate concentration but, especially when the total amount of substrate in a cell is small, a significant amount of it may be sequestered by enzymes, by other macromolecules and supra-molecular structures such as membranes (Sols and Marco, 1970).

It happens that the effects of compartmentation of enzymes and substrates on the regulation of biosynthetic pathways is particularly well illustrated by the arginine pathway in Neurospora crassa (see 1.2.2.1) and thus the cautionary note is particularly relevant to the present work.

Finally, the common assumption that the rates of diffusion of molecules are so high that they can be ignored in the overall enzymatic rate equation will not necessarily be valid where the effective concentrations of substrates are very low (as in the case where the majority of a substrate is normally present as an Enzyme-Substrate complex, e.g. in a multi enzyme aggregate (see Section 1.3.2.1 and Appendix I), and thus effectively sequestered from another potentially utilising enzyme, the rate of the latter enzymic reaction being limited by the rate of diffusion of the substrate from the enzyme aggregate). Furthermore, the diffusion rates of metabolites determined in aqueous solution may be significantly higher than the rates in vivo. This conclusion arises from the realization that the absolute subcellular concentration of soluble macromolecules is very high. Consequently the degrees of freedom of movement of molecules are reduced, effectively imposing a

degree of physical organisation upon the solution. The rates of diffusion of molecules present in very low concentrations may be significantly influenced by this level of organisation (Sols and Marco, 1970).

These criticisms of this particular approach to an understanding of the control of flux through metabolic pathways are meant to suggest that we should be cautious about interpreting the measurements made; the principal criticism of this approach however, is that the identifying criterion is based on unsound assumptions.

1.4.2.3

Interpretation of steady state metabolite concentrations

Net metabolic flow is dependent on reactions being displaced from equilibrium. The extent to which a reaction is displaced, the Disequilibrium ratio (D), is given by the Mass action ratio (M) divided by the Equilibrium constant for the reaction (K_{eq}). The Standard Free energy (ΔG°) of a reaction is expressed as

$$\Delta G^\circ = -RT \ln K_{eq}$$

The Free Energy (ΔG) across a reaction is expressed as

$$\Delta G = \Delta G^\circ + RT \ln M = RT \ln \frac{M}{K_{eq}} = RT \ln D$$

The Free Energy is therefore determined by the Disequilibrium Ratio and must always be negative since M is defined as being less than unity.

In general the overall drop in free energy in going from primary substrate(s) to final product(s) is large; this drop in free energy (or values of D) is distributed unevenly between individual steps in a pathway. It has been argued that metabolic flow can only be 'controlled' through alteration in the activity of enzymes catalysing reactions which are far from equilibrium; if an enzyme catalysed

reaction approaches equilibrium, the forward and reverse rates must be of the same order and hence the enzyme cannot be "limiting" the flow through the pathway. The separation of these reactions which are close to thermodynamic equilibrium ("uncontrollable") from those which are far from equilibrium ("potentially controllable") is somewhat arbitrary although, on quasi-thermodynamic grounds, the latter class may be considered to include all those reactions with D values of less than 0.2. However, identification of an enzyme which catalyses a non-equilibrium step is insufficient evidence to conclude a regulatory role for that enzyme; positive identification can be made however from the determination of intermediate concentrations if the conditions described by the Cross-over Theorem apply. The Cross-over Theorem was originally developed by Chance and his colleagues (Chance et al., 1958) specifically in relation to their observations on the electron transport pathway of coupled mitochondria in the presence of excess oxygen and oxidisable organic substrate but in the presence of limiting amounts of ADP. The key observation was that when respiration became limited by ADP exhaustion, the electron transporting coenzymes close to oxygen became more oxidised while those close to the oxidisable organic substrate became more reduced; at some intermediate position along the electron transporting system there was a "cross-over" in the redox state of the electron-transporting coenzymes.

When the predicted change in flux at the "cross-over" step was deduced from the determined changes in concentrations of oxidised and reduced forms of intermediates it was found to contradict the observed change in flux. This paradox could be explained by assuming that a "flux-controlling interaction" had taken place at the "cross-over" step.

Application of the Cross-over Theorem to the identification of sites in a metabolic pathway at which control of flux occurs is limited by the strict requirements of the theorem: (1) all components of the pathway must be identified; (2) all intermediate components of the pathway must be conserved; (3) the concentrations of each component must be determined. Very few metabolic systems satisfy these stringent requirements; some investigators have been unaware of these requirements or have ignored them. Even so it will only identify sites where a dramatic effect has occurred, following a shift from one metabolic state to another, and will not eliminate the conclusion that less dramatic "interactions" may have simultaneously occurred elsewhere and made their contribution to the change. Furthermore, the identification of an "interaction" site is relevant only to the particular change in metabolic state which leads to its identification and has no predictive value nor any necessary involvement in the response of the same system to a different change in metabolic state.

All three of the above approaches to an understanding of the mechanisms controlling flux through a metabolic pathway imply that control is invariably exerted at a single, or at a few, "sites". In other words that there are "rate-limiting", "rate-controlling" or "pacemaker" enzymes in a metabolic pathway; and that these particular enzymes are of special significance in metabolism. The dubiety of this premise will be seen from a consideration of the analysis of the "Control of Flux" developed by Kacser and Burns (1973 and 1979) which can now be discussed.

The Analysis of the Control of Flux

In this section I will discuss in some detail the analysis of the "Control of Flux" by Kacser and Burns (1973 and 1979) since an understanding of the general underlying thinking behind the theory is intrinsic to the purpose and design of this thesis, the interpretation of the results and to the criticism of other approaches to this general topic.

The components of a metabolic system are divisible into those which are parameters and those which are variables. The parameters represent the constant constraints imposed upon any system (e.g. externally set concentrations of nutrients and genetically determined kinetic characteristics of enzymes) but are generally under our control and may be varied between experiments. The concentration of a particular enzyme may be a variable if, for example, it is subject to induction or repression; or considered to be a parameter if, for example, it is synthesised at a constant rate under the metabolic conditions studied. The concentrations of metabolites are, by definition, variable; it is their characteristic that they move and settle to certain values. At steady state these concentrations are time invariant, their rates of formation being balanced by their rates of removal. The steady state is a system property; the rate of formation and removal of each metabolite pool being dependent on the rates of formation and removal of all metabolically associated pools. These rates are in turn determined by the system parameters; thus the variable metabolic pools provide the links between the parametric system components. In principle, the steady state is a function of all the parameters of the system. Since the fluxes are by definition the flows into and out of the metabolic pools, they too are system

properties. Thus, as has been previously stated, the level of flux in vivo and the mechanisms whereby it is controlled and maintained cannot be deduced solely from analyses in vitro even if such analysis were to encompass all the component enzymes and their kinetic properties.

Two fundamental propositions emerge from the algebraic analysis of enzyme catalysed systems. The first concerns the role of enzyme activities (as determined by their concentration, turnover number and Michaelis constants) in influencing the flux through the pathway in which they act. The second concerns the role of effectors (such as substrates, products, inhibitors and activators) in that same process. Both can only be understood (and experimentally determined) by reference to the method of modulation, i.e. to observe or consider the effect of small movements of some parameter or variable on some other variable. The effect of such modulation is expressed as $\frac{dV/dP}{V/P}$, the fractional change in, say, a flux divided by the fractional change in, say, the concentration of an enzyme. In practice, infinitesimal changes can only be approximated and, therefore, the more useful definition is:

$$\frac{\delta F_j}{F_j} / \frac{\delta E_i}{E_i} \approx Z_i^j$$

This has been named the Sensitivity Coefficient and is a quantitative measure of the "importance" of a particular enzyme, E_i , at the value it has, in influencing the flux, F_j , through a particular pathway (which may be "its own" or any other pathway influenced by changes in that enzyme). It will be realised that, because every pathway is

connected to every other, all enzymes, in principle, affect all pathways, although the quantitative value of the Sensitivity Coefficient will be negligibly small for most.

The Sensitivity Coefficient (which is both algebraically and operationally defined) replaces the grouping of enzymes into "classes" (rate-limiting versus non-rate-limiting etc.) and gives a quantitative value to the measure of "control" exerted.

An important relation between all the Sensitivity Coefficients is established by the Summation Property:

$$\sum_{n=1}^f Z_{E_1}^j = 1$$

This states that the sum of the Sensitivity Coefficients of all the enzymes with respect to one flux is unity. This Property puts a constraint on the value of any one coefficient and, in general, results in the readily observable fact that most enzymes can be reduced in activity (sometimes by large amounts) without any detectable change in flux (see Figure 6). The metabolic fluxes are in principle the phenotype of an organism. The Summation Property accounts for the observation that in diploid organisms most mutations are completely recessive to wild-type; there is no detectable change from the wild-type flux/phenotype in the heterozygote despite there being only half as much enzyme.

Because the coefficients themselves are systemic properties, the same enzyme can have a very low coefficient at one value (e.g. homozygous wild-type) and a high one at another (e.g. homozygous mutant). Its controlling effect can therefore

change with its own concentration or with a change in other elements in the rest of the system. Only modulation over a whole range of conditions can ascertain these aspects.

The second proposition is defined by another coefficient:

$$\frac{dv}{v} / \frac{dS}{S} = \epsilon$$

The Elasticity Coefficient (ϵ) is defined by the ratio of the fractional change in rate of an enzyme catalysed step over the fractional change in some effector (S) causing this change. It is therefore a "local property" describing, quantitatively, the response at one step (and the particular milieu of all substances) to changes in effectors which would be brought about by more distant changes in some other parameter.

Both the Sensitivity and the Elasticity, jointly, influence the change in a flux. This relation is defined by the Connectivity Property:

$$\sum_n z_i \epsilon_s^i = 0$$

This says that the product of the Sensitivity Coefficients with respect to any steps connected by a common "linking" effector (such as a substrate) multiplied by the Elasticity of that effector with respect to the rate of that step, summed over all the connected steps, is zero. This relation really reflects the fact that the "local" Elasticities generate the "global" or systemic Sensitivities and displays, in an algebraically rigorous manner, the close and necessary interactions of all the components of the system. It points to the truth that to understand the behaviour of a system it cannot be "decomposed" into its elements, but must be studied as a whole.

Amino acid transport in *Neurospora crassa*

Before concluding the Introduction mention must be made of aminoacid transport in *Neurospora* since the experimental methodology employed in this investigation relies on the ability to control, by competitive inhibition, the rate of uptake of amino acid(s) required for growth. The description given here will be brief and limited to those points of direct relevance to the experimental approach. A more extensive discussion of aminoacid transport is given in Appendix II to this thesis.

Three distinct, stereospecific transport systems (each identified by single mutations leading to single deficiencies) have been demonstrated to be active in conidia, one specific for the basic aminoacids (Roess and DeBusk, 1968) a second specific for the neutral and aromatic aminoacids (Wolfenbarger and DeBusk, 1971a) and a third with a wide range of specificity (Rao et al, 1975).

At least five different systems are active to varying degrees at different stages in the development from germinated conidia through rapidly growing mycelia to stationary mycelial pads (Pall, 1969, 1970a and b and Railey and Kinsey, 1976). Following the nomenclature of Pall (1969, 1970a and b and 1971) these five systems are: System I, specific to the neutral aminoacids, System II with a general specificity, System III specific to the basic aminoacids, System IV the acidic aminoacids and finally System V is specific for the S-containing aminoacids, L-methionine and L-ethionine.

In the experiments described in this thesis utilising the arg-12 strain L-citrulline was used exclusively as the growth-supporting supplement. This aminoacid is only transported by the

"general" system (Thwaites and Pendyala, 1969). L-histidine, used exclusively as the competitive inhibitor of L-citrulline uptake, is also largely taken up by the "general" system but is also transported via the "basic" system, and to a moderate extent by the "neutral" system (Pall, 1970a and b) L-arginine and L-ornithine, the only other aminoacids employed in this study, are again both taken up by the "general" system. In addition, L-ornithine is taken up by the "basic" system but to a lesser extent than is L-arginine which has a strong affinity for this system (Pall, 1970a and b; Thwaites and Pendyala, 1969).

Mutants, designated mtr, have been selected which are specifically deficient in System I ("neutral") activity (Lester, 1966; Stadler, 1966). Similarly, the bat mutant of Thwaites (1967) is specifically deficient in System III ("basic") activity. The neu^a mutant described by Magill et al (1972) is probably specifically deficient in System II ("general") activity. Other mutants have been described with more general permeation effects (e.g. the un-t mutant of Kappy and Metzenberg (1965) and the nap mutant of Jacobson and Metzenberg (1968)). Finally, in addition to the effect of developmental age, system specific transport activity is also affected by both the mycelial concentration of amino acid ("transinhibition" (Pall and Kelly, 1971)) and the medium concentration of aminoacid ("substrate repression" (Nell and DeBusk, 1974)).

Summary

An abundance of mechanisms are potentially involved in the control of metabolic flux. These are well illustrated by the arginine metabolic pathway in Neurospora crassa. A number of experimental approaches to an understanding of these control mechanisms have been employed in this and other laboratories. A different approach, such as that adopted by the present author, might be expected to be particularly fruitful.

2. MATERIALS & METHODS

2.1 MATERIALS

2.1.1 Neurospora crassa strains: origins and descriptions

The following strains of Neurospora crassa were employed in this study and are held as silica-gel stocks in this laboratory.

- (i) ure a From the silica-gel stocks of Dr. R.Tateson.
Ureaseless
Backcross of original ure-47 mutant of Kølmark (1969a) into the St.Lawrence 74a wild-type silica-gel stock of Dr. C.Curtis.
- (ii) arg-12 ure a From the silica gel stocks of Dr. R.Tateson.
OTCase-less, ureaseless.
Backcross of the original ure-47 mutant of Kølmark (1969a) into the arg-12a strain of Dr. C.Curtis. The latter strain was constructed by back-crossing the OTCase-less mutant, UM 107, of Davis and Thwaites (1963) into the St.Lawrence 74a background.
- (iii) arg-12 ota aga From the silica gel stocks of Dr. I.B.Barthelmess.
OTCaseless, OTAaseless, arginaseless.
This strain was obtained from a cross of arg-12 ure A with aga ota can^s a. The cross was constructed, the segregants isolated and characterised and the genotypes determined by Dr. I.B. Barthelmess.

2.1.2

Chemicals

General chemicals used for making up growth media, extraction mixtures, buffers, enzyme assay mixtures, standards for the calibration of colorimetric methods and reagents for colorimetric assays were all of analytical quality and supplied variously by BDH Chemicals Ltd., Poole, England, Fisons Scientific Apparatus Ltd., Loughborough, Koch-Light Laboratories Ltd., (through A. & J. Beveridge Ltd., Edinburgh, Scotland) and Sigma London Chemical Company Ltd., Poole, England.

Water was always glass distilled.

Radio-chemicals were supplied by the Radiochemical Centre, Amersham, England.

The following radiochemicals were obtained for use as substrates in enzyme assays.

L-(U- ^{14}C) Glutamic acid. Aqueous solution containing 2% ethanol, sterilised $225 \text{ mCi.mmol}^{-1}$

L-(U- ^{14}C) Arginine monohydrochloride. Aqueous solution containing 2% ethanol, sterilised $270 \text{ mCi.mmol}^{-1}$

Reagents used in enzyme assays and containing these radiochemicals were made up as described under Determination of Enzyme Activities (2.2.7).

N.E. 260 (Micellar) Scintillation Fluid was obtained from Nuclear Enterprises Ltd., Edinburgh, Scotland.

2.1.3

Ion exchange resin

All chromatography columns used for the separation of radioactive products from substrates in terminated incubation mixtures (see 2.2.7) were made from Dowex 50 W X 8 (H^+ form 200-400 mesh) after preparative washing and equilibration with the appropriate buffer. The resin was obtained through Sigma London Chemical Company Ltd., Poole, England.

2.1.4

Growth media

Vogel's N liquid medium containing 2% w/v glucose as carbon source and amino acid supplements where applicable was used throughout for growth experiments. Solid medium for the routine maintenance of Neurospora crassa strains was as above with 2% agar (Difco Bacto-agar, Difco Laboratories, Detroit, U.S.A.) added.

Medium was prepared by dilution in distilled H_2O from a stock solution of 50 X N Vogel's salts in distilled H_2O .

Vogel's 50 X N Salt Solution

15 % w/v	tri-sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$).
25% w/v	Potassium dihydrogen orthophosphate (KH_2PO_4).
10% w/v	Ammonium nitrate (NH_4NO_3).
1% w/v	Magnesium sulphate ($MgSO_4 \cdot 7H_2O$).
0.5% w/v	Calcium chloride ($CaCl_2 \cdot 2H_2O$).
0.5% v/v	Trace element solution.
0.025% w/v	Biotin
0.2% w/v	Chloroform

Trace element solution, in distilled H_2O was as follows:

- 5% w/v Citric acid ($C(OH)(COOH)(CH_2.COOH)_2.H_2O$).
- 5% w/v Zinc sulphate ($ZnSO_4.7H_2O$).
- 1% w/v Ammonium ferrous sulphate ($(NH_4)_2SO_4.FeSO_4.6H_2O$).
- 0.25% w/v Cupric sulphate ($CuSO_4.5H_2O$).
- 0.66% w/v Manganese sulphate ($MnSO_4.4H_2O$).
- 0.05% w/v Boric acid (anhydrous) (H_3BO_3)
- 0.05% w/v Sodium molybdate ($Na_2MoO_4.2H_2O$).

2.1.5 "Neurospora extraction mixture" for aminoacid pool analysis

The "mixture" contained exactly $0.4 \mu\text{mol.ml}^{-1}$ L- α -amino- β -guanidino propionic acid (AGPA) in 5% w/v Sulphosalicylic acid (SSC).

2.1.6 Buffers

The following buffers were used in (a) the preparation of mycelial extract supernatants for the assay of enzyme activities or the buffering of enzyme incubation mixtures and (b) the pre-equilibration of, and sample elution from, ion-exchange chromatography columns. All buffers were titrated at R^0T .

(a) (i) 0.05M Tris-HCl buffer, pH 7.5

1 volume of 0.1M Tris-(hydroxymethyl)-aminomethane titrated to pH 7.5 with 0.1M HCl. Diluted to 2 volumes with distilled H_2O .

(ii) 0.5M PO_4 buffer, pH 8.0

$0.5M Na_2HPO_4 + 0.5M NaH_2PO_4$ - titrated to pH 8.0.

(iii) 0.05M PO_4 buffer, pH 7.5

$0.05M Na_2HPO_4 + 0.05M NaH_2PO_4$ - titrated to pH 7.5.

(iv) 0.1M KPO_4 buffer, pH 7.0

1 volume 0.2 M KH_2PO_4 titrated to pH 7.0 with 0.2 M NaOH.

Diluted to 2 volumes with distilled H_2O .

(v) 0.05M Glycine-NaOH buffer, pH 9.5

1 volume 0.1M Glycine titrated to pH 9.5 with 0.1M NaOH.

Diluted to 2 volumes with distilled H_2O .

(b) All buffers used in ion-exchange chromatography were citrate buffers titrated from ^{a sodium or} a sodium/lithium citrate stock buffer to the correct pH with HCl. The final molarity of all buffers was 1.0M. Na Citrate buffer, pH 5.65 also contained 45 g $\text{NaCl} \cdot 1^{-1}$. All other buffers contained 0.2 gm ions. 1^{-1} .

2.1.7 Protein determination reagents

Lowry reagent contained 2% w/v Na_2CO_3 , 0.4% w/v NaOH, 0.2% w/v NaK Tartrate and 0.01% w/v $\text{CuSO}_4 \cdot 4\text{H}_2\text{O}$.

Folin-Phenol reagent was a one-to-one mix of distilled H_2O and Folin-ciocalteau's reagent (BDH Chemicals Ltd., Poole, England).

2.1.8 Colorimetric reagents

The colorimetric reagents used in the methods described in Section 2.2.7.2 and 2.2.8 were as follows.

2.1.8.1 O-aminobenzaldehyde

O-aminobenzaldehyde solution ($3 \text{ mg} \cdot \text{ml}^{-1}$ in 20% ethanol) was used for the colorimetric assay of Δ -PC formed by OTAase activity (see

section 2.2.7.1). The solution was prepared, fresh daily, as follows:

O-aminobenzaldehyde (Sigma London Chemical Co.Ltd., Poole, England) was dissolved in Analar ethanol (BDH Chemicals Ltd., Poole, England). Occasionally a small amount of material remained undissolved after thorough vortexing or a flocculent precipitate was formed on addition of ethanol due to the presence of a small amount of polymerized o-aminobenzaldehyde. Undissolved substances were pelleted by centrifugation (5 min at 4,500 rpm). The supernatant was removed and diluted 5-fold in distilled H_2O to give a working solution containing 3 mg.ml^{-1} O-aminobenzaldehyde in 20% ethanol. (If any additional flocculent precipitate was formed on dilution with H_2O this was removed by centrifugation as before).

2.1.8.2

BUN Reagents

An automated colorimetric assay for urea and L-citrulline is described in section 2.2.8.1. The assay was based on the Blood Urea Nitrogen (BUN) method of Marsh et al, (1965).

The reagents were as follows:

(a) Acid reagent

Acid reagent consisted of 20% v/v H_2SO_4 , 6% v/v H_2PO_4 and 0.0033% w/v $FeCl_3 \cdot 6H_2O$.

(b) Colour reagent

The original colour reagent contained 1.675% w/v Diacetyl monoxime and 0.335% w/v Thiosemicarbazide. It was found that on prolonged standing crystals and precipitates were formed at these concentrations of components with a concomitant reduction in method

sensitivity. It was found that the shelf-life could be significantly prolonged, without loss of method sensitivity or stability of coloured complex, by reducing the component concentrations to 0.25% w/v Diacetylmonoxime and 0.0125% w/v Thiosemicarbazide. In all but some early determinations the later colour reagent composition was used. All the results are, however, intercomparable since a calibration curve from known concentrations of urea and L-citrulline was always constructed for each batch of samples and reagent. This was a precaution rather than a necessity since, except under the conditions alluded to above, the method sensitivity was stable and consistent between reagent batches.

2.1.8.3

PAULY Reagents

An automated colorimetric assay for L-histidine is described in section 2.2.8.2. L-histidine was assayed by means of the diazo reaction using Pauly reagents (Dawson et al, 1959).

The reagents were as follows:

(a) Sodium Nitrite

1.4% NaNO_2 in distilled H_2O .

(b) Sulphanilic acid

0.93% Sulphanilic acid ($\text{NH}_2\cdot\text{C}_6\text{H}_4\cdot\text{SO}_3\text{H}$) in 1% HCl.

2.1.9

Other materials

Filter paper - Whatman No.1, Qualitative filter paper (Whatman Ltd., England) was used exclusively.

Silica gel - 4-6 mesh non-indicating silica gel was obtained from Fisons Scientific Apparatus Ltd., Loughborough, England.

"Parafilm" - sealing tissue was obtained from Gallenkamp,
England.

"Dricold" solid CO₂ was obtained from I.C.I. Ltd., U.K.

2.2 METHODS

2.2.1 Storage and Maintenance of Neurospora crassa strains

Long term storage of strains was achieved by preservation of conidia absorbed on sterile, anhydrous, non-indicating silica gel (Fisons, 4-6 mesh) (Perkins, 1962). Glass tubes (15 x 60 mm) half-filled with silica gel, cotton wool plugged and cork-stoppered were dry sterilised by overnight heating at 120°C. A dense conidial suspension in sterile water was made from 7 to 10 day old agar slant cultures of strains for preservation. Two drops of the conidial suspension with two drops of a sterile 10% w/v solution of commercial dried skimmed milk were added to a sterile silica gel tube. The tube was held in an inclined position and tapped as the drops of solution were added to maximize the distribution of conidia amongst the silica gel crystals and to minimize the heat of absorption (Perkins, 1962). The tubes, clearly dated and labelled with full strain description, were restoppered, recorked and finally sealed with "Parafilm".

The silica gel tube stocks were stored at -15°C. Stocks were checked periodically for viability by tapping out one or two crystals onto appropriately supplemented agar slants. Stocks have been successfully maintained for several years by this method in the laboratory.

Strains were successfully maintained for periods of several months by transferring freshly-conidiated Parafilm-sealed agar-slant cultures from the 29°C incubator to a 4°C constant-temperature room.



Strains in regular use were routinely sub-cultured, at approximately monthly intervals, onto fresh, appropriately supplemented, agar slants and incubated at 29°C.

Strains in current use were sub-cultured onto fresh appropriately supplemented agar slants 7 to 10 days before required for a growth experiment and incubated at 29°C.

2.2.2 Strain identification

The following routine genotype tests were carried out. Their validity was confirmed by the observations on growth behaviour, enzyme activity and metabolic steady states under the experimental conditions described in the text.

2.2.2.1 The ure genotype

The ureaseless genotype was periodically tested by a method based on that of Kølmark (1969b). A clump of conidia from the strain to be tested was placed on a 1 cm long strip of Whatman's BDH indicator paper, narrow range pH 6-8, adhered to the inner wall of a 2 ml plastic test tube and presoaked in a 1% urea solution. The tube was stoppered and incubated at 37°C for 10 minutes. The absence of urease activity is indicated by the absence of a colour change on the filter paper (pale yellow). The presence of urease activity is indicated by a colour change from pale yellow to deep blue (due to the formation of ammonia which causes the pH to become alkaline).

Using this particular indicator paper and with the presoaked paper adhered to the inside of a translucent test tube the test is

simple, reliable and unequivocal. The incubation step is not essential, serving only to standardise the method; a colour change is always observed after a fairly short time at $R^{\circ}T$ with strains having urease activity. In the stoppered tubes the colour change is stable over a period of days.

2.2.2.2 The arg-12 genotype

The ornithine carbamyl transferase-less genotype was routinely tested by the ability to grow on L-citrulline or L-arginine supplemented but not minimal or L-ornithine supplemented agar slants.

2.2.2.3 The arg-12 ota aga genotype

This strain has the same growth requirements as arg-12 but germinates and conidiates comparatively slowly, probably due to a putrescine-limitation resulting from a restriction on endogenous ornithine synthesis. This is an effect of arginine supplementation in combination with the arginaseless mutation (see 1.3).

2.2.3 Mycelial Growth

The procedures adopted for conducting growth experiments with Neurospora crassa were as follows:

2.2.3.1 Preparation of strains for inoculation

Dense conidial suspensions were made from well conidiated agar slant cultures by flooding the slants with sterile H_2O , vortexing vigorously and filtering the suspensions through sterile cotton-wool filters. The concentration of conidia in the filtrate

was determined by dilution and haemocytometer counting. Accurate, identical aliquots of the original or appropriately diluted suspension were added to each growth flask resulting in known inocula, typically of between 5×10^6 and 1×10^7 conidia per flask.

2.2.3.2 Preparation of growth flasks

Conidial suspension were inoculated into 1 l. dimpled, flat-bottomed growth flasks containing 400 ml sterile Vogel's N liquid medium, 2% w/v D-glucose and amino acid supplement where applicable. The growth flasks containing Vogel's medium were sterilised by autoclaving for 20 min at 120°C . Glucose solution was autoclaved separately to avoid caramelization and added, after cooling, to the Vogel's medium under sterile conditions. Aminoacid supplements were made by dissolving in sterile H_2O and adding to the sterilized growth flasks under sterile conditions. This procedure was adopted after it was discovered that certain amino acids decomposed on autoclaving. This method of supplementation never resulted in growth contamination. Aminoacids which were used to compete for uptake with essential aminoacids were generally not added to the growth flasks until at least ten hours after inoculation to circumvent potential inhibitory effects on germination and early growth.

2.2.3.3 Growth rate determination: culture conditions and mycelial harvesting

The inoculated growth flasks were incubated at a constant 29°C with constant agitation (200 rpm) on a New Brunswick gyrorotary shaker rack. Under such conditions the cultures were kept continually

aerated and, at later stages of growth, mycelial clumping was prevented. An exponential increase in mycelial dry weight with time, at least over the range 5 to 500 mg, was observed.

Mycelia were obtained for experimental analysis and, contemporaneously, the growth rate determined by harvesting growth flasks at various times after inoculation. The contents of individual growth flasks were rapidly filtered under suction through two layers of filter paper in a Buchner funnel. The mycelial pad so produced was peeled off, placed in a labelled plastic vial and plunged into an acetone or alcohol/Dricold" (solid CO_2) mixture to achieve rapid freezing. The pads were then vacuum-dried to a pressure of less than 0.01 Torr, weighed and stored at R^oT in a desiccator for subsequent analysis of internal metabolite concentrations and enzyme activities.

2.2.3.4

Medium sampling

The growth media were sampled at various times by drawing 1 to 5 ml volumes through a fine "Millipore" gauze which excluded mycelium from the sample. The medium samples were transferred to plastic tubes and stored at -15°C for subsequent analysis of amino acid supplement and metabolite concentrations.

2.2.4

Preparation of mycelial extracts; homogenisation and centrifugation

Mycelial extracts, whether with the purpose of determining enzyme activities or aminoacid pool concentrations, were prepared as follows, unless specifically stated otherwise. The particular extraction buffer or mixture is stated in the text (see also 2.1.5 and 2.1.6).

All steps were carried out at 4°C . Freeze-dried mycelial powder ($5\text{--}20\text{ mg.ml}^{-1}$) was homogenised with 15 strokes at 5,000 rpm. (TRI-R STIR-R homogeniser, TRI-R Instruments, New York, U.S.A.). The homogenate(s) were centrifuged for 15 min at 4,500 rpm (MSE Super Minor Centrifuge, MSE Ltd., London, England). The supernatants were removed and either assayed for the appropriate enzyme activity or stored at -15°C for subsequent amino acid pool analysis.

2.2.5 Amino acid pool analysis

2.2.5.1 Sample preparation

An accurately known weight of freeze-dried mycelial powder was homogenised in exactly 50 times the weight in volume (i.e. 20 mg.ml^{-1}) of "Neurospora Extraction Mixture" (see 2.1.5). After acid-precipitation of insoluble protein in the cold, the homogenate(s) were centrifuged, the supernatants removed and stored at -15°C for subsequent autoanalysis.

2.2.5.2 Autoanalysis of mycelial extracts

Two amino acid analysers, one a modified Technicon, - the other a modified Locarte - autosanalyser, were used routinely throughout this study. Both used a Ninhydrin based colorimetric detection system and gave consistent and comparable results.

2.2.5.3 Calculation of absolute aminoacid concentrations

Aminoacids present in mycelial extract supernatants were identified by comparison with the elution profile of a standard

mixture containing known amounts of specific aminoacids.

Their absolute concentrations were determined by comparing the aminoacid peak areas with that of the internal standard, AGPA. AGPA was used as the internal standard because (a) it is not a naturally occurring amino acid and (b) it elutes distinctly under the chromatographic conditions employed, from all aminoacids occurring naturally in Neurospora crassa.

The aminoacid concentrations, expressed throughout as $\mu\text{mol.}^{-1}$ 100 mg dry weight, were obtained by multiplying the Area Ratios (aminoacid peak area \div AGPA peak area) by 2 (there were exactly 2 $\mu\text{mol.}$ AGPA per 100 mg dry weight in the mycelial extracts) and finally by an empirically determined "colour value" which takes into account the variation in extinction coefficients for the different Ninhydrin-aminoacid complexes.

2.2.6

Protein determination

The concentration of protein in mycelial extracts was determined by the Folin-Phenol method of Lowry et al., (1951) (see 2.1.7 for reagents). To a sample volume of 0.4 ml, 2.0 ml of fresh Lowry reagent was added with immediate mixing. After standing for 20 minutes at $R^{\circ}T$, 0.2 ml of Folin-Phenol reagent was jetted in with immediate mixing. After standing for 30 min at $R^{\circ}T$ the A_{700} of the samples was determined using a Beckman Double Beam Spectrophotometer (Beckman Instruments Ltd., G.B.).

The protein concentration in samples was estimated from a calibration curve of $\text{Log } A_{700}$ versus $\text{Log } \mu\text{g}$ for accurately known

amounts of bovine serum albumin (BSA, crystallised and lyophilised purest obtained from Sigma London Chemical Company, Ltd., Surrey, England). The standard error in mean A_{700} for duplicate aliquots of BSA was less than 2%. The calibration curve was linear over the range 4-400 μg BSA. The slope was calculated from the regression of $\text{Log } A_{700}$ on $\text{Log } \mu\text{g BSA}$. The standard error in the slope was less than 2%. The sample protein concentration was determined according to Stauffer (1975) from the following formula.

$$\mu\text{g protein in sample} = \frac{A_{700} \text{ sample}}{I} \times \frac{1}{S}$$

where I is the intercept (A_{700} for 1 μg) and S the slope calculated from the regression of $\text{Log } A_{700}$ on $\text{Log } \mu\text{g BSA}$.

2.2.7 Determination of Enzyme Activities

The specific activities of two anabolic and two catabolic enzymes of the arginine pathway were determined in mycelial extract supernatants. The preparation of mycelial extracts has been described (see 2.2.6).

2.2.7.1 Calculation and expression of enzyme specific activities

Initial rates were always measured under conditions of maximal activity (excess substrate and cofactor) and at specified pH and temperature.

All assays relied on the estimation of the amount of product formed in terminated incubation mixtures as detailed under the assay procedures for individual enzymes. Initial rates were calculated from the regression of product formed on time. The standard error of the slope was consistently less than 10%.

Protein concentration in mycelial extracts was determined

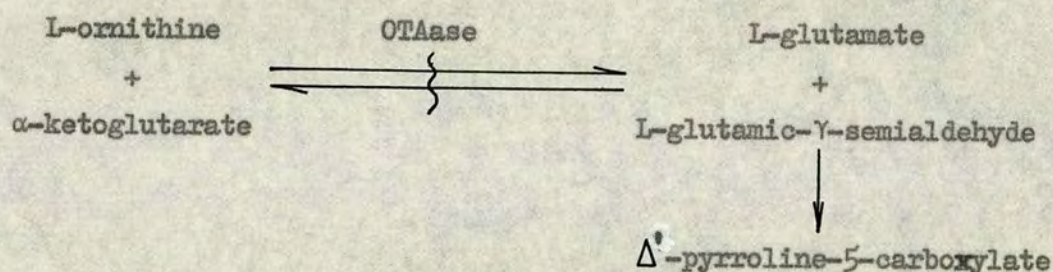
as described in 2.2.6. The A_{700} of duplicate aliquots of mycelial extracts were determined. The standard error of the mean was always less than 1% and usually less than 2%.

Enzyme specific activities were universally expressed as nmol product formed. $\text{mg}^{-1} \text{protein} \cdot \text{min}^{-1}$ at a specified pH and temperature.

2.2.7.2

Ornithine transaminase (OTAase) (L-ornithine: 2-oxoacid aminotransferase, E.C.2.6.1.13).

The reaction catalysed is as shown below (see also Figure 1).



Enzyme activity in mycelial extract supernatants was determined by a method based on that of Vogel and Kopac (1960) and Dr. S. Baumberg, personal communication. Transamination from the amino group of L-ornithine to α -ketoglutarate proceeds virtually to completion due to the spontaneous cyclization of the product, L-glutamic- γ -semialdehyde (GSA) to Δ^5 -pyrroline-5-carboxylate ($\Delta^5\text{PC}$). The assay relied on the reaction of this last compound with o-aminobenzaldehyde to form a deep yellow dihydroquinazolinium derivative with a maximum absorption at 443 nm and a millimolar extinction coefficient of 2.71 (Jenkins and Tsai, 1970 and Davis and Mora, 1968).

Assay procedure

Freeze-dried mycelial powder was homogenised in 0.1M KPO_4 buffer, pH 7.0 (approximately 5 mg.ml^{-1}). The homogenate was centrifuged and the supernatant removed and assayed for enzyme activity.

The incubation mixture contained the following:

0.05 ml Mycelial extract supernatant in 0.1M KPO_4 buffer, pH 7.0.

0.25 ml 0.5M PO_4 buffer, pH 8.0.

0.05 ml 0.01M Pyridoxal phosphate - fresh, in distilled H_2O .

0.20 ml 0.05M α -ketoglutarate - fresh, in distilled H_2O .

0.05 ml 0.2M L-ornithine.HCl - in distilled H_2O .

The reaction was initiated by the addition, with mixing, of mycelial extract supernatant. The mixtures were incubated for 0, 20, 40 and 60 min at 37°C .

The reactions were terminated by the addition, with mixing, of 1.2 ml, 0.5N HCl to the incubation mixtures.

Two minutes before the reactions were terminated, 0.2 ml of O-aminobenzaldehyde solution (3 mg.ml^{-1} in 20% ethanol, see 2.1.8) was added, with mixing, to the incubation mixtures.

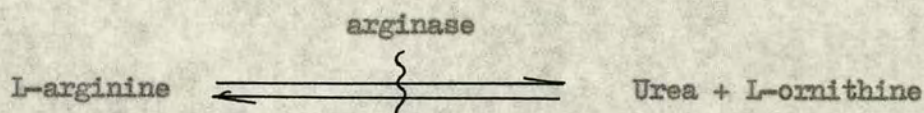
Estimation of product formed

The A_{440} of undiluted, terminated incubation mixtures was measured in a Beckman Double Beam Spectrophotometer or, after appropriate dilution, by a Technicon-detection system. The amount of ΔPC formed (assumed to be the same as the amount of enzyme product, GSA, formed) was calculated from the known millimolar extinction coefficient of the dihydroquinazolium derivative (see above).

2.2.7.3

Arginase (L-arginine ureohydrolase, E.C.3.5.3.1)

The reaction catalysed is shown below (see also Figure 1).



Enzyme activity in mycelial extract supernatants was determined by a method based on that of Middelhoven (1964) and Davis and Mora (1968). The original method of Middelhoven (1964), as with several other published methods (e.g. Messenguy *et al.*, 1971) included an enzyme activation step. No activation step was included by Davis and Mora (1968) nor was it by the present author. The principal aim here was not to determine maximum enzyme activities but to develop a simple, reliable and repeatable method for determining initial rates. This aim was achieved by adopting the assay procedure to be described. The assay relied on the determination of the reaction product Urea, by the BUN method (see 2.2.8.1).

Assay procedure

Freeze-dried mycelial powder was homogenised in 0.05M TRIS-HCl buffer, pH 7.5, approximately 5 mg.ml^{-1} . The homogenate was centrifuged and the supernatant removed and assayed for enzyme activity.

The incubation mixture contained the following:

0.1 ml Mycelial extract supernatant, in 0.05M TRIS-HCl buffer, pH 7.5.

0.5 ml 0.05 M Glycine-NaOH buffer, pH 9.5.

0.1 ml 1.0 M NaCl, in distilled H_2O .

0.1 ml 0.005 M $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, in distilled H_2O

0.2 ml 0.125 M L-arginine. HCl, in 0.05 M Glycine-NaOH buffer, pH 9.5.

The reaction was initiated by the addition, with mixing, of mycelial extract supernatant. The mixtures were incubated for 0, 5, 10, 15 and 20 min at 30°C . Either a complete incubation mix was used for each time point, the reaction being terminated by the addition, with mixing, of 0.2 ml 30% Sulphosalicylic acid (SSC) or successive 0.2 ml aliquots were removed from a single incubation mixture with time and added, with mixing, to 0.2 ml. 0.2 N HCl. In the former case, zero time points corresponded to an incubation mixture where SSC had been added before mycelial extract supernatant; in the latter case, to aliquots removed and added to HCl immediately after initiation of the reaction.

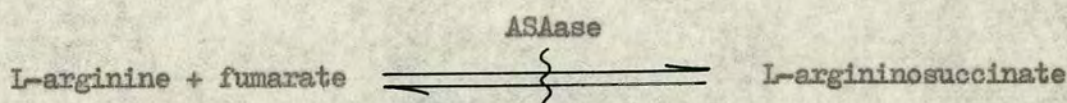
Estimation of product formed

It was shown by column chromatography that the total BUN-positive material accumulated in terminated reaction mixtures over that present in zero time point mixtures was urea. The rate of product (i.e. urea) formation was calculated from the determined extinction coefficient at A_{520} for urea complexed with BUN reagents and the rate of accumulation of total BUN positive substances determined at the same wavelength (see section 2.2.8.1 for description of BUN method).

2.2.7.4

Argininosuccinase (ASAase) (L-argininosuccinate
arginine-lyase, E.C. 4.3.2.1)

The reaction catalysed is as shown below (see also Figure 1)



The reaction has a K_{eq} . of the order of unity (Ratner, 1970); depending on the concentrations of reactants the reaction rate can be conveniently measured in the forward or reverse direction. (Forward is used in the sense of the overall direction in vivo, i.e. in the direction of L-arginine and fumarate).

Enzyme activity in mycelial extract supernatants was determined by an isotopic method based on that of Kato et al, 1976. They drove the reaction in the reverse direction; their method relied on the chromatographic separation and determination of L-(^{14}C) argininosuccinate, formed from the substrate (U- ^{14}C)-fumaric acid, in terminated reaction mixture. In the present method L-(U- ^{14}C) arginine HCl was used as substrate; all other factors being equal this method is preferred since it is simpler and easier to separate and elute ASA from ARG rather than ASA from FUM chromatographically.

Assay procedure

Freeze-dried mycelial powder was homogenised in 0.05 M PO_4 buffer, pH 7.5 (approximately 10 mg.ml^{-1}). The homogenate was centrifuged and the supernatant removed and assayed for enzyme activity.

The incubation mixture contained the following:

0.1 ml Mycelial extract supernatant in 0.05M PO_4 buffer, pH 7.5.

0.1 ml 0.06M Na fumarate in 0.05M PO_4 buffer, pH 7.5.

0.1 ml 0.06M L-(U- ^{14}C)-arginine. HCl ($0.01 \mu\text{Ci.}\mu\text{mol}^{-1}$) in 0.05M PO_4 buffer, pH 7.5.

The reaction was initiated by the addition, with mixing, of L-(U- ^{14}C)-arginine HCl. The mixtures were incubated for 0, 20, 40 and 60 min at 37°C . The reactions were terminated by the addition, with mixing, of 0.05 ml 30% sulphosalicylic acid (SSC). In the case of zero time points, SSC was added before L-(U- ^{14}C)-arginine HCl.

Estimation of Product formation

After acid precipitation of protein in the cold, the terminated incubation mixtures were centrifuged and the total supernatants carefully loaded onto Dowex 50 X 8-400 resin chromatographic columns (5 X 40 mm), pre-equilibrated with Na citrate buffer, pH 5.0. The sample was allowed to drain through the column under gravity. The sample was followed through with Na citrate buffer, pH 5.0, the first millilitre of which was used initially to "wash" the precipitated protein pellet in the sample tube. The first 1.35 ml. of column eluent were discarded and the two succeeding 1 ml fractions collected directly into scintillation vials each containing 10 ml NE 260 (Micellar) scintillant. (It had established, by taking successive, small eluent fractions from the time of loading a sample, that at least 95% of the enzymatically-derived counts were recovered in the 1.35 to 3.35 ml fraction of the column eluent). The equivalence of "counts" and "ASA" was confirmed by the aminoacid autoanalysis (see 2.2.5) of eluent fractions; all the Ninhydrin-detectable ASA was eluted in the 1.35 to 3.35 ml fraction and in the absence of any detectable ARG.

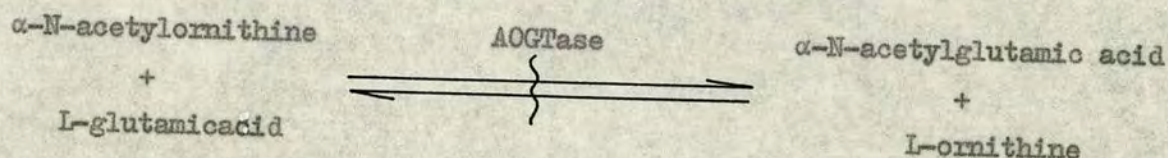
The scintillation vials were counted in a Nuclear Chicago Isocap 300 scintillation counter on the channel appropriate for ^{14}C . The counts measured were corrected for machine counting efficiency by comparison with an internal ^{14}C standard. The d.p.m.

were converted to $\mu\text{moles ASA}$ from the known input specific activity of $\text{L-(U-}^{14}\text{C)-arginine HCl}$.

2.2.7.5

Acetylornithine glutamate transacetylase (AOGTase)

The reaction catalysed is as shown (see also Figure 1).



Enzyme activity in mycelial extract supernatants was determined by a method based on that of Denes (1970). The assay relied on the chromatographic separation and determination of $\alpha\text{-N-(}^{14}\text{C)-acetylglutamic acid}$, formed from the substrate $\text{L-(U-}^{14}\text{C)-glutamic acid}$, in terminated incubation mixtures.

Assay procedure

Freeze-dried mycelial powder was homogenised in 0.05M TRIS-HCl buffer pH 7.5 (approximately 5 mg.ml^{-1}). The homogenate was centrifuged and the supernatant removed and assayed for enzyme activity.

Two alternative assay procedures were employed.

(i) Incubation mixtures contained the following:

- 0.1 ml Mycelial extract supernatant in 0.05M TRIS HCl buffer, pH 7.5.
- 0.2 ml 0.05M TRIS-HCl buffer, pH 7.5.
- 0.1 ml 0.15M $\alpha\text{-N-acetylornithine}$, fresh, in 0.05M TRIS-HCl buffer, pH 7.5.
- 0.1 ml 0.15M $\text{L-(U-}^{14}\text{C)-glutamic acid}$ ($0.033 \mu\text{Ci.}\mu\text{mol}^{-1}$) in 0.05 TRIS-HCl buffer, pH 7.5.

The reaction was initiated by the addition, with mixing, of $\text{L-(U-}^{14}\text{C)-glutamic acid}$. The mixtures were incubated for 0, 20, 40 and 60 min at 37°C . The reactions were terminated by the addition, with

mixing, of 0.5 ml 0.2N HCl. In the case of zero time points, HCl was added before L-(U- ^{14}C)-glutamic acid.

(ii) Incubation mixtures contained the following:

0.20 ml Mycelial extract supernatant in 0.05M TRIS-HCl buffer, pH 7.5.

0.20 ml 0.05M α -N-acetylornithine, fresh, in 0.05 M TRIS-HCl buffer, pH 7.5.

0.20 ml 0.15M L-(U- ^{14}C) glutamic acid ($0.033\mu\text{Ci}.\mu\text{mol}^{-1}$) in 0.05 M TRIS-HCl buffer, pH 7.5.

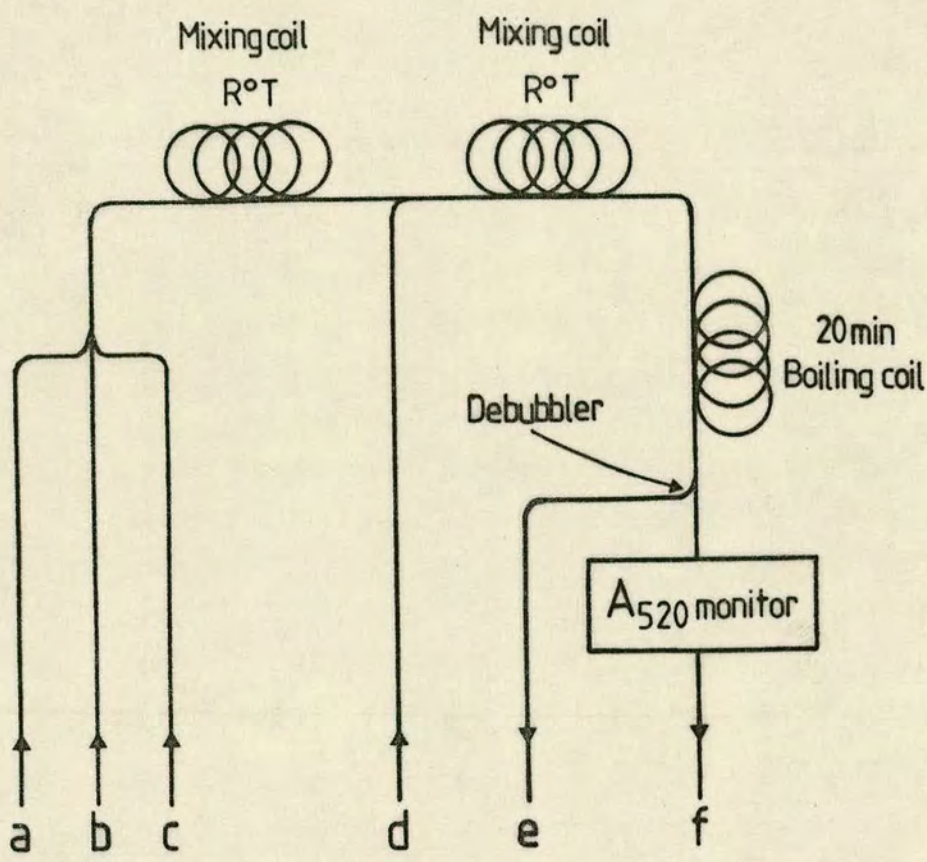
0.25ml 0.05M Tris-HCl buffer, pH 7.5.

The reaction was initiated by the addition, with mixing, of L-(U- ^{14}C)-glutamic acid. Successive 0.2 ml aliquots were removed at 0, 20, 40 and 60 min from the reaction mixture, incubated at 37°C , and added with mixing, to 0.2 ml, 0.2N HCl. In the case of the zero time points a 0.2 ml aliquot was removed and added to HCl immediately after the addition of L-(U- ^{14}C) glutamic acid.

Estimation of product formation

After acid-precipitation of protein in the cold, the terminated incubation mixtures were centrifuged and the total supernatants carefully loaded onto Dowex 50W X 8-400 resin chromatographic columns (5 X 40 mm), pre-equilibrated with Na citrate buffer, pH 2.0. The sample was allowed to drain through the column under gravity. The sample was followed through with 0.1 N HCl, the first millilitre (in the case of Procedure (i)) or the first half-millilitre (in the case of Procedure (ii)) of which was used initially to "wash" the precipitated protein pellet in the sample tube. The first 0.5 ml of column eluent was discarded and the two succeeding 1 ml fractions collected directly into scintillation vials each containing 10 ml NE 260 (Micellar) scintillant. (It had been established, by taking

Figure4. An automated colorimetric method, using BUN reagents, for the determination of urea and L-citrulline.



<u>Flow line</u>		<u>Flow rate (ml per min)</u>
a	Sample	0.16
b	Air	0.60
c	BUN Colour reagent	0.42
d	BUN Acid reagent	0.60
e	Air removal	0.92
f	Developed sample	0.80

successive, small eluent fractions from the time of loading a sample, that more than 90% of the enzymatically-derived counts were recovered in the 0.5 to 2.5 ml fraction of the column eluent).

The scintillation vials were counted in a Nuclear Chicago Isocap 300 scintillation counter on the channel appropriate for ^{14}C . The counts measured were corrected for machine counting efficiency by comparison with an internal ^{14}C standard. The dpm were converted to $\mu\text{moles } \alpha\text{-N-acetyl-ornithine}$ from the known input specific activity of $\text{L-(U-}^{14}\text{C)-glutamic acid}$.

2.2.8 Automated Colorimetric Methods

2.2.8.1 An automated method for the determination of urea and L-citrulline

An automated, colorimetric method, based on the Blood Urea Nitrogen (BUN) test of Marsh et al (1965) and adapted from Technicon Auto-analyser Methodology N-1c, was developed for the determination of Urea and/or L-citrulline. Solutions analysed included medium samples, mycelial extract supernatants and terminated enzyme assay reaction mixtures.

The reagent compositions are described in section 2.1.82.

The method is illustrated in Figure 4.

The sample stream (a) was first segmented with air (b) and mixed with colour reagent (c). After next mixing with acid reagent (d) the single air-segmented stream was developed colorimetrically by passing through a boiling coil. The coil length was sufficient for 20 min boiling. The colour developed stream was debubbled (e) and the

absorbance continually monitored at 520 nm by a Locarte or Technicon detection system (f).

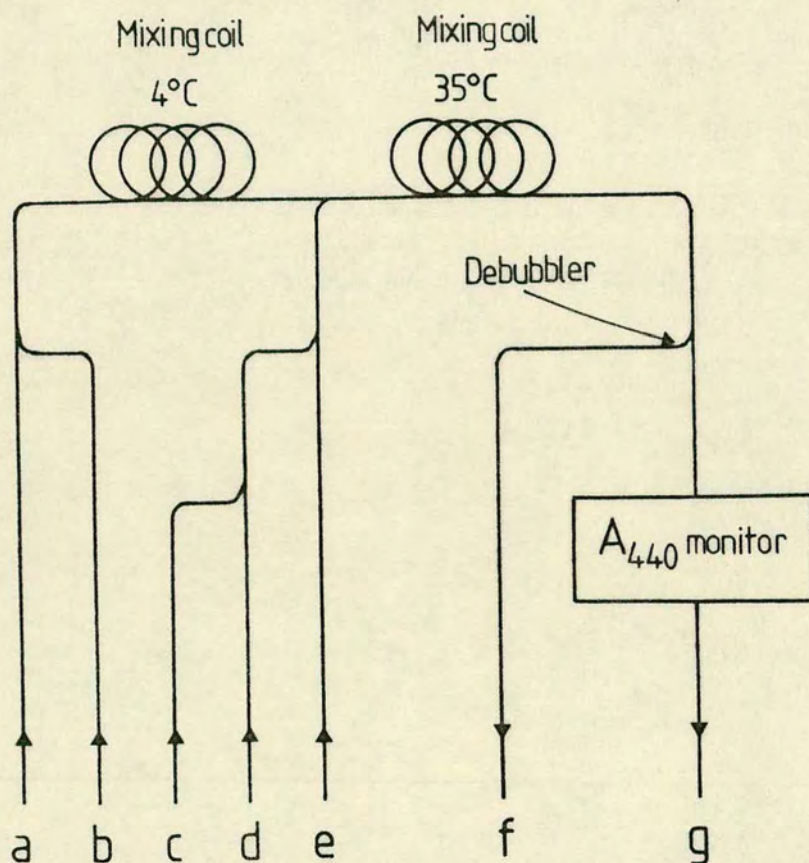
The sample stream either derived from an automatic sampling machine or consisted of part or all of the column eluent from a modified Locarte amino acid autoanalyser chromatogram.

The automatic sampler alternated sample with H_2O so that successive sample peaks on the recorder were clearly separated. The peak height (A_{520}) was shown to be linearly related to concentration for solutions containing known amounts of urea or L-citrulline. This method determines the total BUN-positive substance in a sample. It was used when samples contained only urea or L-citrulline or when, between related samples (e.g. terminated arginase assay reaction mixtures, see 2.2.7.3), one substance remained constant while the other varied in concentration.

The urea and L-citrulline were separated chromatographically in samples where both were present and variable. Separation and elution was achieved using a modified Locarte aminoacid autoanalyser. Samples, in Na/Li citrate pH 2.2 buffer, were absorbed onto the top of the column, pre-equilibrated with Na/Li Citrate buffer, pH 2.8. Urea and L-citrulline were separated and eluted by 25 min 0.2M LiOH followed by 125 min Na/Li citrate buffer, pH 2.8 at $50^{\circ}C$ and a flow rate of 0.2 ml.min^{-1} .

Urea was eluted after about 30 min of the chromatogram; L-citrulline was eluted after about 100 min. The peak areas were shown to be linearly related to quantity for known amounts of urea and L-citrulline. A longer chromatogram, where the LiOH was preceded by 300 min of Na citrate buffer, pH 5.65 was employed when L-histidine was also present, as in medium samples. The latter

Figure 5. An automated colorimetric method, using Pauly reagents, for the determination of L-histidine.



<u>Flow line</u>	<u>Flow rate (ml per min)</u>
a Sodium nitrite	0.16
b Sulphanilic acid	0.16
c Sample	0.16
d Sodium hydroxide	0.16
e Nitrogen	0.32
f Nitrogen removal	0.42
g Developed sample	0.55

compound was determined, by a different colorimetric method (see section 2.2.8.2) concomitant with urea and L-citrulline, after splitting the chromatographic column eluent between the two automated colorimetric methods. The proportions of the column eluent serving as "sample" for each automated colorimetric method was determined by the relative amounts of urea and L-citrulline to L-histidine in the particular sample. The proportionation was achieved by means of an adjustable cam, driven by a constant speed motor, alternatively opening and closing the flow to each sample line.

2.2.8.2

An automated method for the determination of L-histidine

An automated colorimetric method, employing Pauly reagents (Dawson et al, 1959) for the determination of L-histidine, was developed. Solutions analysed included medium samples and mycelial extract supernatants.

The reagent compositions are described in section 2.1.8.3.

The method is illustrated in Figure 5.

The colorimetric reaction only takes place under alkaline sample conditions. The sample stream (c) was first mixed with 1.5N NaOH (d), segmented with N_2 (e), then introduced into a stream of sodium nitrite (a) and sulphanilic acid (b) previously mixed at 4°C. The combined N_2 -segmented flow was mixed and incubated for 10 minutes at 35°C to permit colour development and facilitate degassing of the mixture. The colour developed stream was then debubbled (f) and the absorbance continually monitored at 440 nm by a Locarte or Technicon detection system (g).

The method was primarily developed with the aim of determ-

ining the concentration of L-histidine in medium samples, which was believed to be the predominant, if not the only, Pauly-positive substance in the medium. Analysis of total Pauly-positive substance in medium samples gave spurious results. The reason for this was revealed on chromatogramming medium samples using a modified Locarte aminoacid auto-analyser and developing the column eluent by the method described above; D-glucose (as does sucrose) reacts with the reagents to give a strong A_{440} . Relatively small amounts of other unidentified substances were also detected. These were not positively identified but were probably imidazoles. Their amounts were too small for quantitative analysis.

A chromatogram was devised which eluted L-histidine clearly and separately from all other reactive compounds. The chromatogram has been described in connection with an automated colorimetric method for the determination of urea and L-citrulline (2.2.8.1). L-histidine was eluted after about 300 min of the chromatogram. The peak area was shown to be linearly related to quantity for known amounts of L-histidine.

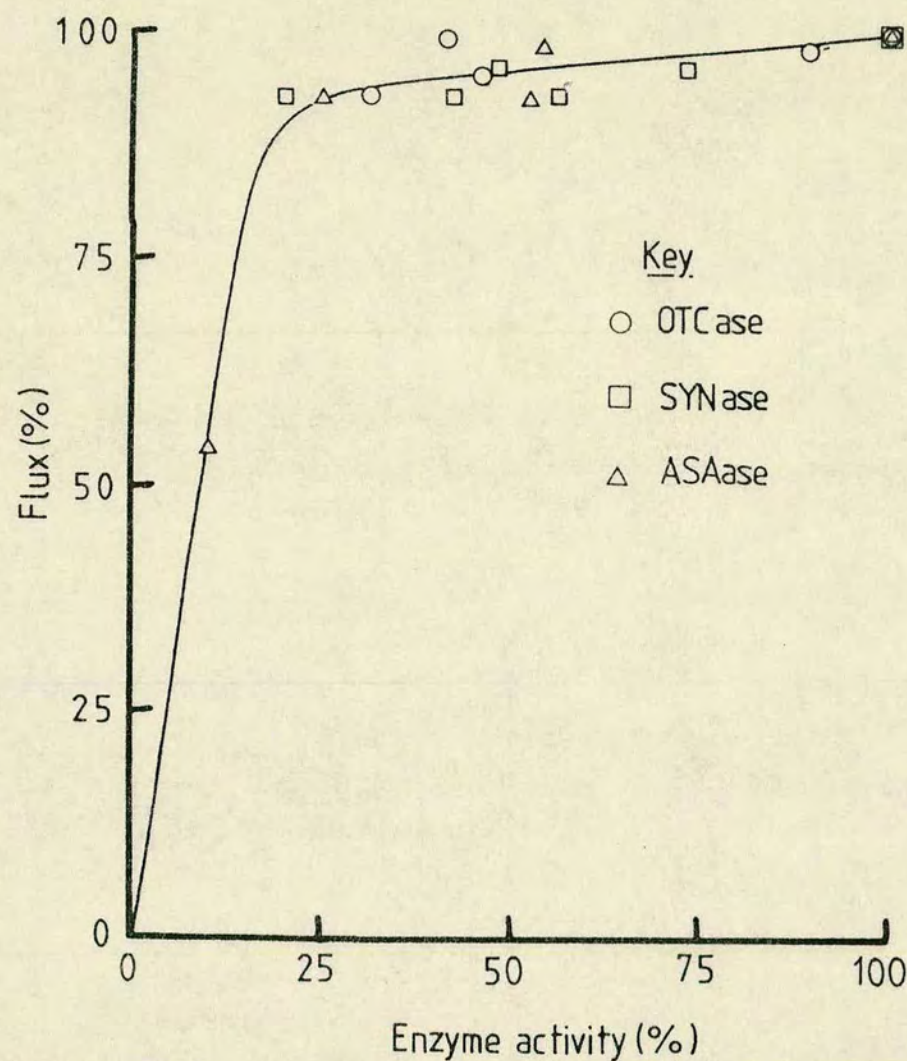
2.2.9 Determination of pH of solutions

Merck pH indicator strips were used for the approximate titration and pH determination of buffers and reagents. Whatman-BDH narrow range pH 6-8 indicator papers were used specifically in a test for the ureaseless genotype in Neurospora crassa (see 2.2.2.1).

Quantitative titration of buffers and accurate pH determinations of solutions were always carried out using a digital pH meter (PHM 52) with combined glass/reference electrode (Ag/Ag Cl filled cells) supplied by Radiometer, Copenhagen. The pH meter was calibrated with electrometrically checked buffer solutions supplied by BDH chemicals Ltd., Poole, England.

Figure 6: The relation between enzyme activity and the flux through the arginine pathway.

Data from Tateson (1971), reproduced with the kind permission of the author. See accompanying text for details.



3. THE DEVELOPMENT OF THE EXPERIMENTAL METHODOLOGY

3.1. The historical background

To set the background to the present study the two previous attempts in this laboratory at a quantitative analysis of flux through the arginine pathway in Neurospora are worthy of mention.

Watson (1969) and Tateson (1971) constructed heterokaryons of Neurospora containing different ratios of wild-type to null mutant alleles at loci coding for biosynthetic enzymes of the arginine pathway. The amount of extractable enzyme formed by a heterokaryon was shown to be directly proportional to the input of wild-type alleles over a wide range of ratios. They were therefore able to correlate the known dosage of wild-type allele or the determined specific activity of the coded enzyme with the rate of urea accumulation by the cultures, a direct measure of the flux through the arginine pathway. Their experimental observations were consistent with the general predictions of the Kacser and Burns (1973 and 1979) analysis of the control of flux. They found that the activity of each enzyme could be reduced substantially below that observed in wild type with no significant reduction in the metabolic flux through the pathway (Figure 6). In other words the Sensitivity Coefficients of the wild-type enzymes were universally low. Only when an enzyme activity was drastically reduced compared with that in wild-type Neurospora did the pathway flux begin to respond significantly, or in other words, did the Sensitivity Coefficient of the enzyme, at the level it had, approach unity. At the same time it was observed that the proportion of gene dose/enzyme concentration began to deviate from unity. At very small wild type/null mutant ratios derepression of biosynthetic enzymes in the hetero-

karyons were observed. This correlated with reduction in the mycelial level of arginine to values below that observed in wild-type Neurospora grown on minimal medium (Tateson, 1971). Barthelmess et al., (1974) greatly extended this quantitative, systemic approach using revertants (bradytrophi) instead of heterokaryons. They introduced several different mutant alleles at each of the three loci which code for the enzymes catalysing the last three steps in the pathway leading to arginine biosynthesis. Each of the alleles coded for enzymes with reduced but measurable activity compared with that observed in wild-type grown on minimal medium, as determined in vitro. Each of the strains with their various combinations of mutant and wild-type alleles were prototrophic and grew on minimal medium. The direct consequence of each combination of mutant and normal enzymes was to generate around themselves a characteristic steady state of metabolites which varied between the various strains with different combinations of mutant and normal alleles. When the activities of the mutationally unaltered enzymes were determined, various degrees of derepression were observed compared with the activity levels characteristic of wild-type Neurospora grown on minimal medium. A strong negative correlation was found to exist between the degree of derepression of the three enzymes and the total extractable intramycelial arginine concentration. The activity of enzymes, however, showed no derepression when these same strains were grown on medium supplemented with arginine or citrulline and when the mycelial arginine pool was as high or higher than the wild-type pool under the same conditions. When the steady state level of ARG fell to below that characteristic of wild-type minimally grown cultures

derepression of enzyme activity was observed. A sharp, up to five-fold derepression was observed with a change in the arginine pool from approximately 0.5 to 0.1 $\mu\text{mol } 100 \text{ mg}^{-1} \text{ d.wt.}$ (Figure 3).

Partial correlations between derepression ratios and other metabolite concentrations were additionally discernible, but these were all explicable in terms of the metabolic relationship between arginine and other intermediates. The relationship between the extractable mycelial arginine concentration and the degree of enzyme derepression follows a sigmoid curve. This relationship is wholly consistent with the belief that the biosynthetic enzymes of the arginine pathway are subject to an allosteric repression system for which the intramycelial concentration of arginine is the "signal" and in which arginine or some directly related derivative acts as the co-repressor (Barthelmess et al, 1974; Burns and Kacser, 1977).

It has already been stressed that for a quantitative analysis of the control of flux any attempt at interpretation of experimental data requires that the biological system under study is at or approximates closely to a metabolic steady state. We can be confident that this criterion is satisfied in the strains analysed in the aforementioned study of Barthelmess et al, (1974). Firstly, all the strains grew exponentially with normal doubling times on Vogel's minimal medium. Furthermore, the amino acid pools were substantially time invariant in all the cultures under all of the growth conditions.

The study of Barthelmess et al, (1974) employed a variety of strains grown under several culture conditions covering an extensive range of metabolic pool levels and enzyme specific activities. Consequently their interpretation of the results is based on a series

of discontinuous data from different steady state conditions. It would be highly desirable to generate a continuous variation in the signal level and to determine simultaneously the levels of related intermediary metabolites and enzyme activities in a single experimental system and to compare the results with those from the aforementioned study. Additionally, information on the metabolic fluxes and their relationship with the arginine (or other) pool level and enzyme activities would be an essential element to an understanding of the control of flux. This objective could, in principle, be approached by continually extending the methodology adopted by Barthelmess et al (1974), namely to produce and introduce further combinations of alleles coding for a further variety of enzyme activities and thus generating a further variety of metabolic steady states. The selection, isolation, characterisation and introduction of the appropriate mutants, however, is a difficult and time consuming occupation.

An alternative and potentially more profitable approach would be to attempt to control the intramycelial arginine pool by external means, namely by controlling the rate of uptake of exogenously supplied arginine, or a precursor, in an arginine requiring strain. Tateson (1971) attempted to do just this. The citrulline requiring strain arg-12 was inoculated into medium containing different ratios of citrulline to histidine. The rationale was that since these two aminoacids competitively inhibit each others uptake in Neurospora (Pall, 1969 and 1970a), the intramycelial level of citrulline, and consequently that of arginine, could be controlled by controlling the external ratio of histidine to citrulline and hence the rate of uptake of citrulline. However, Tateson (1971) was never able to achieve

arginine pools in exponentially growing cultures lower than that characteristic of wild-type minimally grown cultures which we have seen to be necessary for derepression. Furthermore, even at low ratios of histidine to citrulline in the medium the growth of cultures was frequently retarded.

Flint (1977) readdressed the question as to whether control of the intramycelial arginine pool size could be achieved effectively by the competitive inhibition of uptake of an essential exogenously supplied aminoacid. In the light of the apparent toxic effects of histidine observed by Tateson (1971), Flint (1977), incidental to his study of compartmentation, experimented with glycine, another competitive inhibitor of citrulline uptake (Pall, 1969 and 1970a), again employing the arg-12 strain. He found that the mycelial citrulline pool, and consequently that of arginine, could be predictably set at different steady state levels by controlling the exogenous ratio of glycine to citrulline. However, as Tateson, (1971) had observed with histidine, a toxic effect of glycine was clearly indicated. That high molar concentrations of glycine alone in the medium had a toxic effect on the growth of wild-type Neurospora was suggestive that the effect was one of glycine per se rather than an indirect effect through the imposition of a growth limiting rate of uptake of citrulline. The growth rate data relevant to the glycine effect, although not conclusive, were consistent simply with a retardation of germination rather than an increase in the doubling time of the cultures. Flint (1977) found that the toxic effects of both histidine and glycine could be largely obviated if their addition was delayed until approximately eight hours

after inoculation (into minimal medium in the case of wild type Neurospora or citrulline supplemented medium in the case of arg-12).

In summary, previous workers have established, through genetic manipulation of the amount or activity of pathway enzymes, firstly, the relationship between the level of arginine pool and the level of derepression of biosynthetic enzymes (Barthelmess et al, 1974) and, secondly, some information on the relationship between enzyme activity and flux through the pathway (Watson, 1969 and Tateson, 1971). Making use of the ability to control the mycelial arginine pool by the competitive inhibition of citrulline uptake the experimental manipulation of the system can now be extended.

The Experimental Strategy

Having described the historical background to the present work it remains only to state the objectives of this research and the experimental approach which was adopted before proceeding to the presentation of the results.

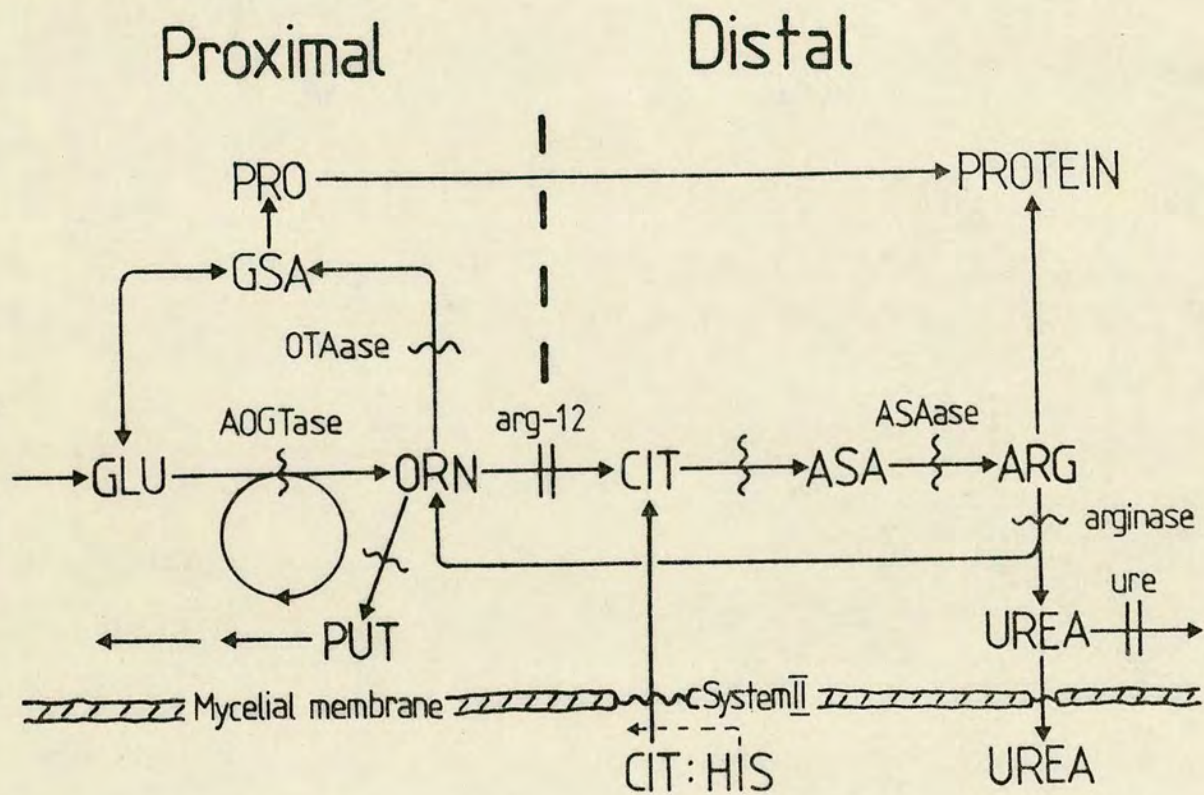
The principal objective of this project was to develop and utilise a method whereby the intramycelial concentration of arginine could be controlled and varied by external means and to monitor, analyse and interpret the effects, if any, of such modulations on enzyme specific activities, metabolic fluxes and other metabolite concentrations. A considerable array of evidence, from a variety of experimental approaches, analyses and experimenters, implicates ARG as a primary effector in the regulation of the activities of enzymes of both its synthesis and degradation (see for example Barthelmess et al, 1974; Cybis and Davis, 1974 and 1975 and Weiss and Anterasian, 1977). With the notable exception of the study by Barthelmess et al (1974), however, the literature is virtually devoid of quantitative analyses of the control and regulation of the arginine pathway in Neurospora, notwithstanding the extensive study of this area of metabolism.

Control of the mycelial arginine pool size in an arg-12 strain was achieved by competitively inhibiting, with histidine, the rate of uptake of exogenously supplied citrulline. Histidine, rather than glycine, was preferred as the competitive inhibitor for the following reasons:

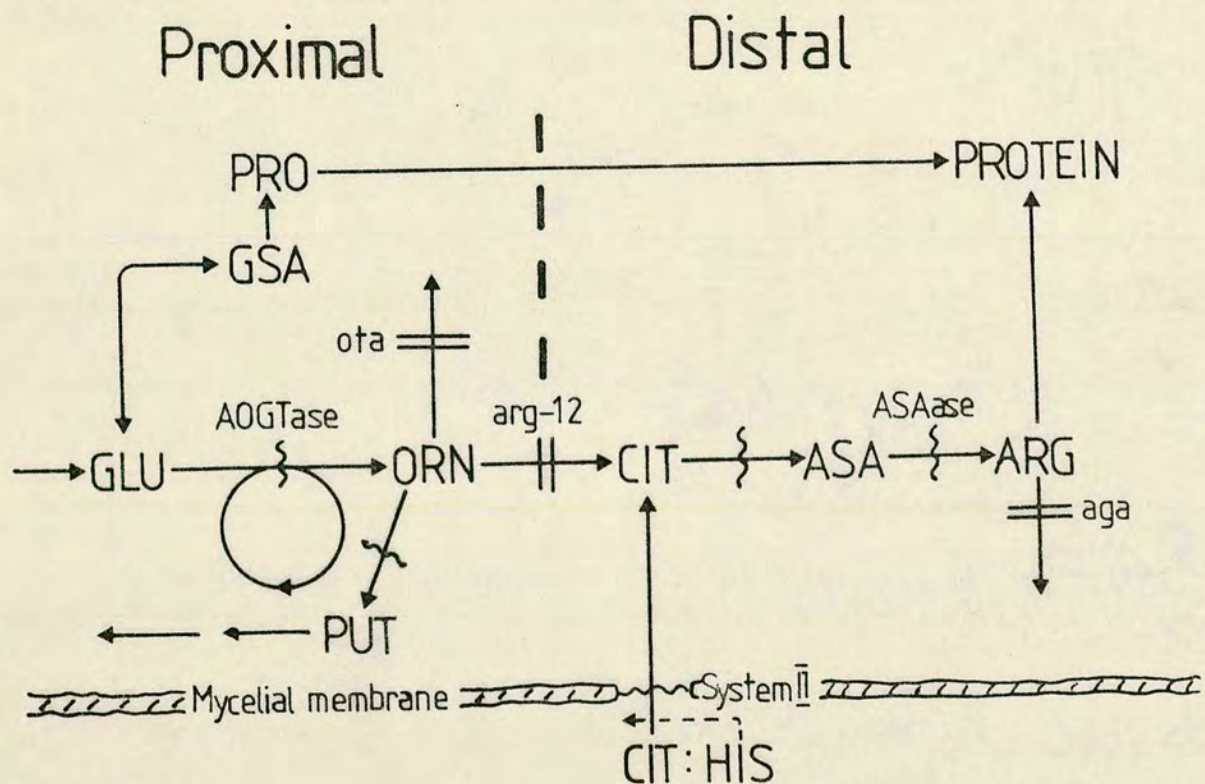
(a) The minor toxic effects of histidine on growth were found by Flint (1977) to be less mole for mole than those of glycine.

Figure 7. The experimental system; the proximal and distal moieties of the pathway.

a) The arg-12 ure system.



b) The arg-12 ota aga system.



(b) Histidine chromatograms clearly and separately on the autoanalyser from all other aminoacids normally found in Neurospora mycelial extracts. In contrast, glycine chromatograms immediately after citrulline. As a result, when present in high concentrations, glycine interferes with the determination of citrulline, particularly when the concentration of the latter is relatively low (as would generally be the case were glycine employed as a competitive inhibitor of citrulline uptake).

As already mentioned, all the intermediates, as well as products, of the arginine pathway are experimentally accessible permitting steady state values and fluxes to be estimated. Assays for the relevant enzymatic steps have also been developed. As well as imposing an arginine requirement and, therefore, the potential for controlling the mycelial arginine pool, the arg-12 mutation has other consequences which could be exploited in a study of the control of flux through the pathway. The block at OTCase breaks the metabolic connection of the steps proximal to CIT from those distal to it (Figure 7). This means that the (external) control of CIT uptake totally replaces the normal endogenous supply from ornithine. On the other hand, in the arg-12 ure strain, the presence of arginase, as well as being a "catabolic" enzyme for ARG, produces ORN thus adding to the endogenous production from GLU (Figure 7). Additionally, the presence of OTAase constitutes a flux removing some of the ORN. A second strain, arg-12 ota arg, lacking both these latter enzymes, was also investigated. A comparison with arg-12 ure would disclose their contributions, both positive and negative, to the ORN flux.

In the arg-12 ota aga strain all metabolic connections between the two moieties of the pathway are severed (Figure 7). There remains, however, an indirect effect of the distal moiety on the proximal through the operation of feedback inhibition and derepression by the (distal) ARG. Thus changes in the flux and hence the "end product", ORN, of the proximal moiety can be studied by modulating the "end product" of the distal part, ARG, acting as a signal.

The design of experiments to carry this into effect fall into four areas:

(a) The germination/growth inhibition by histidine had to be investigated further to establish optimum conditions for maintaining an exponential steady state. It was clear from the work of Tateson (1971) and Flint (1977) that the time of addition of HIS was critical. A series of experiments with varying times of post-inoculation addition were therefore carried out and these are reported in Section 4.1.

(b) The range of HIS:CIT ratios in the medium giving an appropriate range of mycelial ARG pools had to be established. The approximate range of ratios was already known from the work of Flint (1977) but these did not extend significantly into ^{the} "derepression" range of ARG pools. A series of experiments establishing these conditions are reported in Section 4.2.

(c) Methods or modifications of methods to determine all relevant pools and fluxes had to be devised. These methods are described in Section 2.2. The aminoacid autoanalyser would give directly the levels of GLU, ORN, PRO, CIT, ASA, ARG and HIS. In addition to the values for the "pathway" intermediates, the

values of the "unrelated" pools LYS and GLY were examined as indicators of the steady state condition. After separation by column chromatography the concentrations of urea and CIT could be determined in mycelial or medium samples by the BUN method. Similarly, the depletion rate of HIS from the medium could be determined by the PAULY method.

(d) An appropriate choice of enzymes to cover the effects on the pathway had to be made. Inspection of Figure 7 shows that argininosuccinase (ASAase) is a suitable enzyme to monitor the derepression of the "distal" half of the pathway. It will be remembered that the "coordinate" derepression of this part of the pathway has been well established (Barthelmess et al, 1974) (See Figure 3). Additionally, the biosynthetic enzyme, acetylornithine glutamate transacetylase (AOGTase), was chosen to monitor the derepression of the "proximal" part (Cybis and Davis, 1975). By comparison with ASAase, the overall degree of coordinacy of the pathway derepression could be established.

Arginase was chosen for study since not only does this enzyme contribute to ORN but it was also known to be inducible by ARG (Canedo et al, 1967). Finally, and for similar reasons; ornithine transaminase (OTAase), forming the link to the proline pathway (Figure 7), was chosen as this enzyme also contributes to the fluxes maintaining ORN in the "proximal" part and is inducible by ARG (Canedo et al, 1967).

4. RESULTS AND DISCUSSION

The experiments to be described in this Section divide conveniently into three sub-sections. In the first, the effects of HIS on growth will be described and discussed. In the second, the steady state arg-12 ure data will be presented. In the third and final sub-section the "run down" and "run up" condition will be described and the conclusions which may be drawn from its analysis will be discussed.

Most of the discussion of the results will be conducted at the conclusion to each sub-section. The wider implications of the results and their relation to the work of other investigators will be introduced where appropriate. A synthesis of the factors shown by the experimental analyses performed to effect the control of flux through the pathway will be given at the conclusion to the Section.

The complete experimental details and results of analyses performed are given in a table for each experiment which for ease of reference have all been placed together at the end of the thesis in Appendix III. Figures, employing data extracted from one or more table to illustrate particular points revealed by the experimental results, are interspersed within the text at the most appropriate point.

4.1 Growth Inhibition by Histidine

The following series of experiments were conducted to determine the effect of the time of HIS addition on the growth and metabolic state of arg-12 cultures and thus the optimum conditions for establishing an appropriate range of ARG pools under steady state conditions through the inhibition of CIT uptake by HIS.

4.1.1 Experiment I: the germination effect of HIS on arg-12 strains

The experimental design is outlined and results presented in Table 1 (Appendix III).

Conidia of arg-12 ure or arg-12 ota aga were inoculated into medium supplemented with 0.4 mM CIT plus various concentrations of HIS. The initial HIS:CIT ratios ranged from 2:1 to 7.5:1, in steps of 0.5. Duplicate flasks were used for both strains and each HIS:CIT ratio. After 24 h incubation, growth was only observable in those flasks which had been inoculated with arg-12 ure into initial medium HIS:CIT ratios of 2:1 or 2.5:1. Indeed, no growth was detectable under the conditions of higher HIS:CIT ratios even after 42 h incubation. The harvest yields under the 2:1 HIS:CIT regime were substantially lower than would have been observed had the cultures been grown on CIT supplement alone. (Extrapolating from the relevant growth curve from Experiment VII a (Figure 13) and adjusting for the difference in conidial inocula the CIT-only 24 h yield would have been about 500 mg). The yields from the 2.5:1 HIS:CIT cultures were lower still and significantly different between duplicate flasks. In those cultures which grew, the ARG pools were, as predicted, substantially less than those observed in cultures grown on CIT supplement alone but never less than that character-

istic of minimally grown wild type cultures. These results, therefore, confirm the findings of Tateson (1971) that even at low HIS:CIT ratios the addition of HIS at the time of inoculation has considerable effects on growth. Furthermore, although affecting the pool level, ARG values in the derepression range were not achieved. This indicated that if successful experiments were to be conducted, under conditions of rapid exponential growth, cultures would have to be incubated for a certain time in medium supplemented with CIT alone.

4.1.2

Experiment II: the post-germination effect of HIS on *arg-12* ure

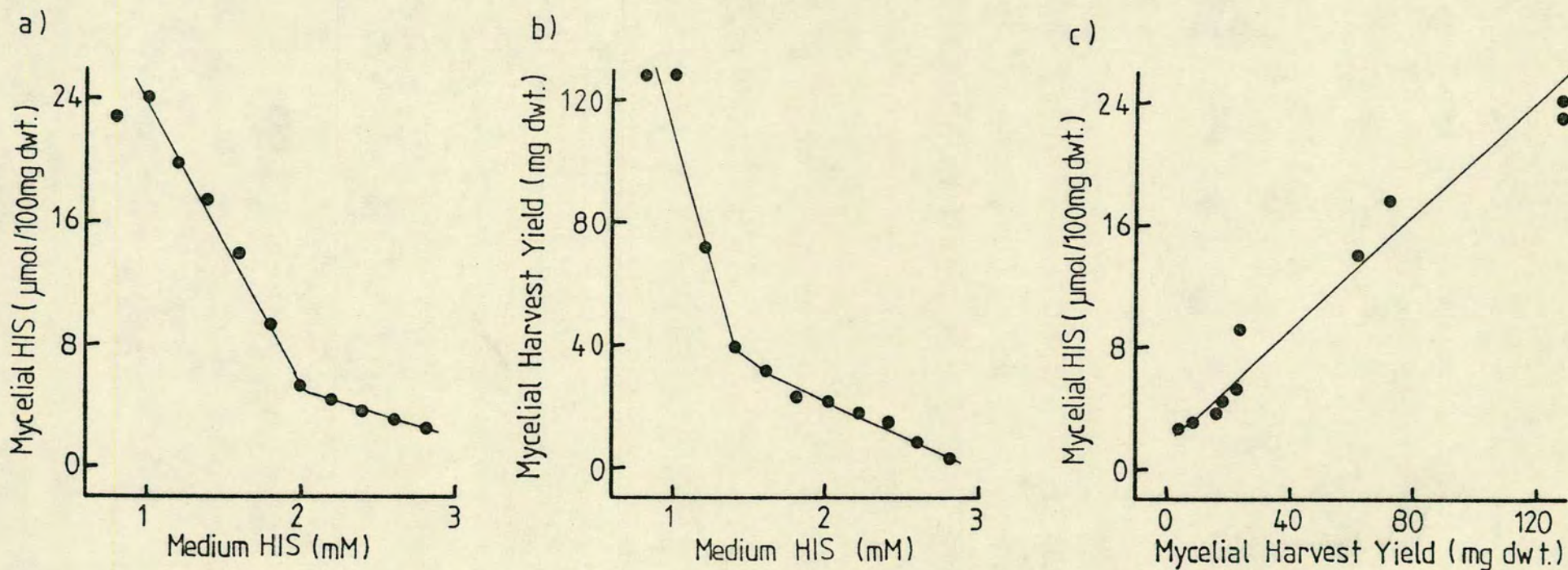
The experiment was conducted in essentially the same manner as Experiment I (Table 1), but the addition of HIS was delayed until 8 h after the inoculation of the conidia into CIT supplemented medium.

Only those cultures grown in HIS:CIT ratios of 2:1 or 2.5:1 had harvest yields of the order expected for the same conidial inoculum incubated solely in the presence of CIT. The harvest yields under each condition of HIS:CIT ratios greater than 2.5:1 were significantly and progressively lower. This indicates that even when addition is delayed until 8 h after inoculation HIS has an inhibitory effect on growth. Moreover, this effect is concentration dependent.

As predicted, the CIT pool, and consequently the ARG pool, falls with increasing HIS:CIT ratio with the latter pool falling to well within the derepression range at ratios greater than 3:1. However, the serious, concomitant growth effects mean that the 8 h delay in HIS addition is still too short for our purpose. However, the results in themselves merit further comment.

Figure 8: Experiment II: the effect of medium HIS on the mycelial harvest yield and HIS pool.

Data from Table 2, Appendix III. See accompanying text for details.

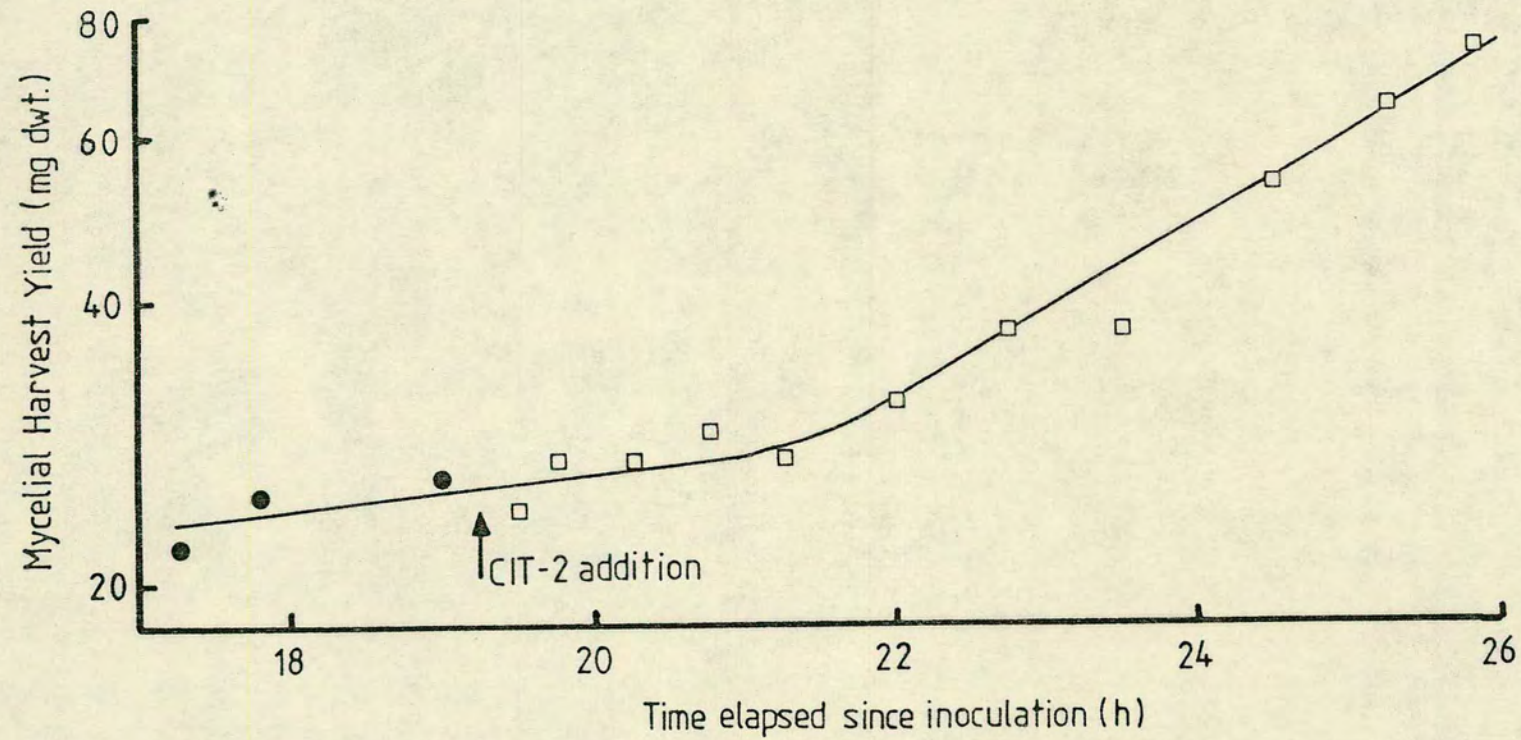


The first surprise comes when the mycelial HIS pool is plotted against the external HIS concentration (Figure 8a). An apparently paradoxical result is obtained; the higher the external concentration the lower the mycelial pool. Almost exactly the same form of relationship is observed when, instead of the mycelial HIS pool, the harvest yield is plotted against the external HIS concentration (Figure 8b). (It should be stated that the biphasic curves drawn in Figures 8a and b are not intended to imply (a) biphasic process(es)). A possible mechanism which could explain these correlations is disclosed in Figure 8c which shows a substantially linear relationship between harvest yield and the mycelial HIS pool. (The linear relationship is "improved" still further when it is realized that the cultures grown under the 5 highest HIS:CIT ratio were harvested significantly later and therefore their yields are proportionately elevated compared with those cultures grown under lower ratios of HIS:CIT). This linear relationship suggests that the rate of HIS uptake is a function of the mycelial "age", as reflected in the harvest yield. "Age" is not meant here to represent chronological time but the developmental stage of the fungus. Thus the strongly inhibited cultures (high HIS) have developed and grown more slowly and it is suggested that the development of a HIS transport system takes place pari passu with growth and development. This concept is in accord with the study of Railey and Kinsey (1976) on the development of transport activity.

The possibility that the growth inhibition is mediated via the inhibition of CIT uptake is not borne out by the mycelial pool data (Table 2); the CIT and ARG values are comparable to or higher than the

Figure 9: Experiment III: growth rate.

Data from Table 3 , Appendix III. See accompanying text for details.



wild-type strain or bradytroph values with perfectly normal growth (Barthelmess et al., 1974). At the same time the mechanism of HIS inhibition cannot be via the internal HIS concentration since the greatest inhibition is shown with the lowest internal HIS pools. We are therefore left with the speculation that the HIS effect must act "externally", possibly through a concentration dependent action on the formation, integration or function of some membrane(s) vital for development.

Several predictions arise from these speculative hypotheses. Firstly, the later the HIS addition, the more "mature" the cultures will be and therefore the lower will be the growth inhibitory effect of HIS for a given concentration (or HIS:CIT ratio). Similarly, the later the addition of a given ratio of HIS:CIT the higher will be the corresponding mycelial HIS pool. These and other expectations were examined in the following series of experiments.

4.1.3

Experiment III: the reversibility of HIS-mediated growth inhibition

In this experiment the reversibility, or otherwise, of the HIS growth inhibition effect was analysed. The details of the experimental design and the results, are presented in Table 3. Briefly, a series of flasks, supplemented with CIT only, were inoculated with arg-12 ure conidia, incubated for 7 h 15 min then HIS added to each ^{to} yield a HIS:CIT ratio of 5:1, incubated for a further 12 h and finally more CIT added to bring the HIS:CIT ratio down to about 1:1.

The mycelial harvest yields are plotted against the time elapsed since inoculation in Figure 9. Clearly, the growth rate is

very low for the cultures in a 5:1 ratio of HIS:CIT (the mycelial doubling time is approximately 20 h). However, the doubling time falls to a respectable 3 h 15 min within 3 h of the addition of more CIT and the reduction of the HIS:CIT ratio to about 1:1. The HIS inhibition effect is, therefore, completely reversible. This reversibility must mean that the HIS growth effect is not solely concentration dependent but also dependent upon the HIS:CIT ratio. The implications of this observation on the nature of the HIS effect will be discussed at the conclusion to this sub-section.

4.1.4

Experiment IV: the post-germination effect of HIS on arg-12 ota aga

This experiment was conducted in essentially the same manner as Experiment II (Table 2) except that the arg-12 ota aga (as opposed to arg-12 ure) strain was used and, most importantly, the addition of HIS was delayed until 10 h 15 min after the inoculation of conidia into CIT supplemented medium. The details of the experimental design and the results of the analyses are presented in Table 4. Note that two different conidial inocula were used depending on the ratio of HIS:CIT established. This was done to compensate for expected effects on the yields at higher HIS:CIT ratios and must therefore be taken into account when comparing yields obtained with different inoculi.

The most striking difference between this experiment and Experiment II is that here the effect of increasing HIS:CIT ratio on harvest yield is substantially removed, with less than a 50% drop (as compared with over 95% drop) in harvest yield for an increase in HIS:CIT ratio from 2.1 to 7:1. This is assumed to be a direct consequence of delaying the addition of HIS from 8 h to 10 h 15 min

after inoculation. Comparison of the results from Experiments I and II with the observations made here provides strong evidence for the efficacy of HIS-mediated growth inhibition being directly related to the developmental age of the culture at the time of HIS presentation.

The pool data also support the contention that the development of HIS transport activity takes place pari passu with growth and development; the mycelial HIS pools observed here are without exception higher than the same pool at corresponding HIS:CIT ratios in Experiment II (Table 2). This elevation in the HIS pool with increasing delay in the time of HIS addition is most noticeable at the higher ratios of HIS:CIT.

Again, the CIT pool, and consequently the ARG pool, fall with increasing HIS:CIT ratio, with the values of the latter pool extending well into the derepression range at HIS:CIT ratios of 4.5:1 and above.

4.1.5

Experiment V: the effect of PUT on growth inhibition

The purpose of this experiment was two-fold; firstly, to determine the effect on growth and pools of delaying the addition of HIS still further and, secondly, to examine what effect, if any, the addition of PUT to the medium had on the growth of both the arg-12 ure and arg-12 ota aga strain. The logic behind posing the second question was as follows:

In Experiment I a complete lack of growth of arg-12 ota aga conidia was observed (Table 1). This might have been a direct effect of the HIS added to the medium at the time of inoculation. Alternatively,

the cause may have been a limiting flux to putrescine (PUT). PUT, which is essential for growth, can only be formed in vivo through the decarboxylation of ORN. The explanation of the differential effects of the same treatment of arg-12 ure and arg-12 ota aga in Experiment I may reside in the difference in sources of ORN between the two strains. In arg-12 ota aga ORN can only be formed from acetylated precursors. As will be shown later in the thesis, the endogenous biosynthesis of ORN is subject to both inhibition and repression by the mycelial ARG pool. Thus if conidia in liquid CIT supplemented medium accumulate high mycelial ARG pools, irrespective of exogenous HIS, then the lack of growth of arg-12 ota aga would be explicable in terms of an ARG effect on ORN biosynthesis and a consequential deficiency in PUT. In support of this notion the germination, growth and conidiation of this strain on ARG supplemented solid agar slants is relatively poor and slow. Although the growth of arg-12 ure is seen to be affected by HIS (Tables 1, 2 and 4) we would not expect a PUT-starvation effect since in this strain ORN can be formed catabolically from ARG in addition to biosynthetically from acetylated precursors.

Details of the experimental design and the results are presented in Table 5.

In the event, no significant difference was observed in either strain between the harvest yields with or without PUT added for the same HIS:CIT ratios. The reason for the complete lack of growth of the arg-12 ota aga cultures in Experiment I must remain unresolved.

However, a HIS effect on growth is still in evidence, though clearly the effect is less, at equivalent HIS:CIT ratios, than in earlier experiments where the post-inoculation delay prior to HIS addition was shorter (Tables 1, 2 and 4).

4.1.6.

Summary and Conclusions

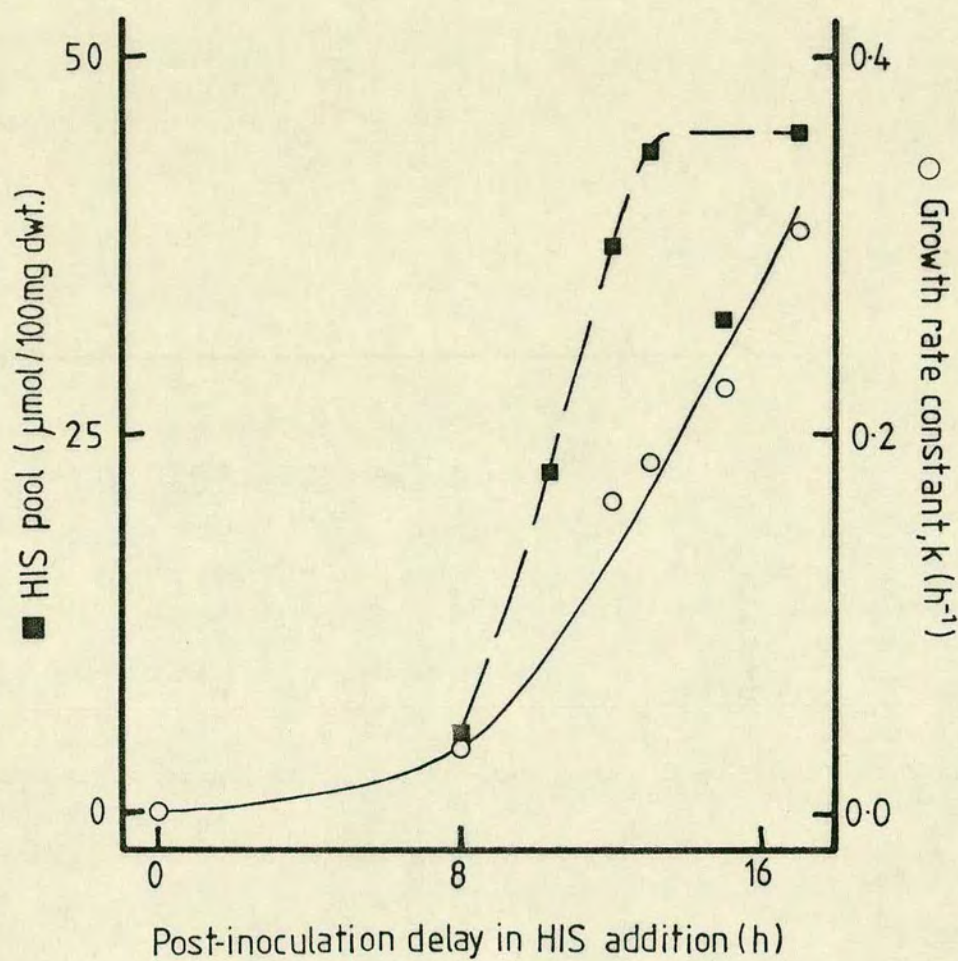
The following observations can be made from the results of the preceding experiments:

- (a) The growth inhibitory effect of HIS is dependent on the developmental age of the cultures at the time of HIS presentation (compare Tables 1, 2 and 4).
- (b) Development of HIS transport activity takes place pari passu with growth and fungal development (compare Tables 2 and 4 and see Figure 8c).
- (c) The HIS effect on early growth and development of transport activity is dependent not only on the time of HIS addition but also the absolute concentration of HIS added (Figure 8a).
- (d) Dramatic growth inhibition is observed despite the presence of an ARG pool substantially higher than those of many bradytrophic strains with normal exponential growth (Tables 1, 2, 4 and 5, Barthelmess et al., 1974).
- (e) Although the ARG pools observed argue against ARG-starvation being the cause of growth inhibition, completely normal exponential growth can be restored by the addition of more CIT to the medium (Figure 9).

We can add profitably to these observations certain results from experiments yet to be described which bear directly upon the effect

Figure 10: The effect of the post-inoculation delay in HIS addition on the growth rate and the mycelial HIS pool.

Data from Table 6, Appendix III. See accompanying text for details.



of HIS on growth and development.

Firstly, the double effect of the time of HIS presentation on a) the growth rate and b) the development of HIS transport activity can be demonstrated clearly by plotting the growth rate constant and the level of mycelial HIS attained (where these components are determinable) against the time between inoculation and HIS addition at a constant ratio of HIS:CIT. These data from all the experiments where a 5:1 ratio was employed are detailed in Table 6 and plotted in Figure 10. The figure strongly indicates that the development of HIS transport activity is directly related to the developmental age of the cultures and that growth, in general, and the development of HIS transport activity, in particular, are very sensitive to HIS, especially in the first 8 h or so after inoculation. There is an indication that development of HIS transport activity is approaching a maximum at 15 h after inoculation. At 17 h there is no longer a detectable effect on growth rate (see growth rate of CIT-only cultures in Experiment VIIa, Figure 13).

Secondly, in addition to the HIS effect on germination and early growth there is also an effect on late growth observed as a reduction in the exponential growth rate after prolonged growth in the presence of HIS. This effect is dependent upon the HIS:CIT ratio employed (Figures 11 and 13).

Taking all these observations together a plausible explanation for the HIS effects is as follows:

Firstly, there is a direct effect of the external HIS concentration on fungal development and consequently the development of the HIS transport system. The observation that the system shows

greatest sensitivity to HIS during the first 8 h after inoculation (corresponding to the time of germination, germ tube formation and primary hyphal branching) supports the belief that the effect is at the level of mycelial membrane development.

Secondly, there is an indirect effect of HIS on growth through the inhibition of ARG translocation across the vesicular membrane. The basic observations behind this hypothesis are (a) the HIS:CIT ratio dependent reduction in growth rate with prolonged incubation in the presence of HIS, (b) the inhibition of growth despite the presence of a significant ARG pool and (c) the reversibility of growth inhibition by CIT. These three observations can be explained if HIS alters the normal distribution of mycelial ARG such that despite the presence of a relatively large total extractable pool the pool of ARG available for protein synthesis can become growth rate limiting. Furthermore, these observations suggest that this abnormal distribution is caused by the inhibition of ARG translocation across the vesicular membrane by HIS; the egress of ARG within the vesicle prior to the addition of HIS is inhibited as is the vesicular uptake of CIT-derived cytosolic ARG.

In the absence of label exchange studies, we have no direct evidence for or against this hypothesis. There are several pieces of independent evidence which favour its operation. Firstly, we know that HIS and ARG are taken up at the mycelial membrane by a common uptake system (Pall, 1970a). Secondly, we have evidence that HIS, like ARG, is normally largely sequestered to the vesicle (Ferrier, 1978). Finally, we know that ORN and ARG inhibit each

other's vesicular uptake, probably through competition for a common carrier complex (Davis et al, 1978b). The argument for HIS inhibition of ARG translocation across the vesicular membrane is, therefore, strong.

This working hypothesis will be seen to be applicable to a number of unexpected and disparate observations made in the experiments now to be described. In addition, certain expectations which arise directly from the hypothesis will be shown to be borne out by the experimental results.

4.2 The "Steady State" Condition

In the previous section it was shown that the effects of HIS on germination and growth could be largely obviated if the addition of HIS were delayed until at least 10 h after inoculation of the arg-12 strain into CIT-supplemented medium. The data also suggested that by varying the medium HIS:CIT ratio so the mycelial CIT pool could be varied in a predictable way, and consequently the ARG pool set at levels from above to well within the "de-repression" range. The following series of experiments were conducted to determine the effect of varying the ratio of medium HIS:CIT on the steady state levels of mycelial aminoacid pools, in particular that of ARG, and the corresponding effects, if any, on the specific activity of enzymes involved in the arginine pathway, as outlined in Section 3.2.

4.2.1 Experiment VIa: Mycelial harvest yields and aminoacid pools in arg-12 ure.

The experimental design is outlined and the results of the analyses performed are presented in Table 7 (Appendix III). The sixteen growth flasks were divided into four sets of four, each set having a different initial concentration of added HIS. In anticipation of possible growth inhibitory effects the conidial inocula were varied, the higher HIS:CIT ratios, (c) and (d), receiving double the inoculum of (a) and (b). In the event, the anticipated effect was overcompensated (see Yields in Table 7). After 13 h incubation on medium supplemented with CIT alone HIS was added to each set of flasks to yield the initial concentrations detailed in Table 7. After a further 6 to 7 h incubation, flasks within each set were harvested at successive one to two hourly intervals. The resultant mycelial were freeze dried and weighed, to

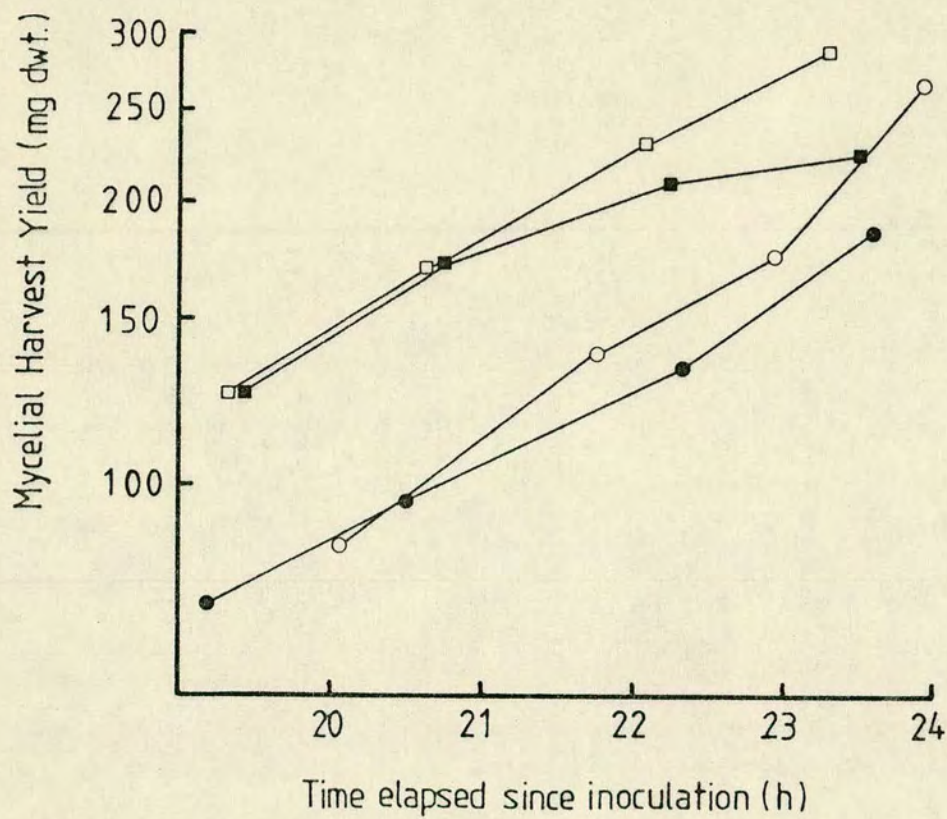
Figure 11: Experiment VIa: growth rate.

Data from Table 7 , Appendix III.

See accompanying text for details.

KEY

Symbol :- ○ ● □ ■
Flask no. :- (a) (b) (c) (d)



give the dry weight yields, then stored, desiccated at room temperature for subsequent aminoacid pool analysis. The later analysis was performed as described in Section 2.2.5. The concentration of CIT remaining in the medium was estimated in samples taken immediately before each flask was harvested by an automated colorimetric method employing BUN reagents, as described in Section 2.2.8.1.

The following general observations can be made and conclusions drawn from the experimental data as presented in Table 7 and plotted in Figures 11 and 12.

(i) Growth rate

The logarithm of the harvest yields are plotted against time in Figure 11. A positive straight line slope from such a plot is indicative of exponential growth. Although the data are insufficient for any rigorous quantitative statements to be made, a slight effect of HIS on early growth is indicated. Apart from the deviation from exponentiality at later time points under condition (d), the highest level of HIS supplementation, the growth rate is much the same for all cultures. The yields from (c) and (d) compared with (a) and (b) at equal, early time points are slightly below the expected factor of two on the basis of inocula presumably reflecting a remaining effect of HIS on growth even at 13 h post-inoculation addition. The departure from exponential growth at later time points under condition (d) may be explained by the hypothesis expounded in Section 4.1.6, namely that HIS inhibits the translocation of ARG across the vesicular membrane. In this case the degree of inhibition must be such that, despite a relatively high total ARG pool, the flux into the cytosolic ARG pool becomes insufficient to support the flux to protein-ARG necessary for exponential growth.

(ii) Mycelial aminoacid pools

The following general observations ^{can be} made and conclusions drawn from the intramycelial aminoacid pool data, presented in Table 7.

(a) In general, the levels of CIT, ARG and ORN are substantially constant within each culture condition although there is a tendency for the CIT and ARG pools to fall and the ORN pool to rise with increasing time elapsed since HIS addition. As will be discussed later, this reflects the slow adjustment of the system towards the "true" steady state. But cultures within sets are at a quasi-steady state since the relaxation times of the metabolic conversions are very much faster than the slow pool changes observed under the experimental conditions employed.

(b) In any case the variation in pool sizes within sets is much less than the variation between sets of cultures; the mean level of mycelial CIT, and consequently that of ARG, falls progressively with increasing ratio of HIS:CIT. In contrast, the mycelial HIS pool is substantially the same under all conditions suggesting that the rate of HIS uptake is the same (though not its competitive effect on CIT), and therefore presumably saturated, under all levels of supplementation employed.

(c) The data from within and between sets of cultures reveal two important correlations between pools. Firstly, there is a strong positive correlation between the CIT and ARG pools. This is to be expected in view of their direct metabolic relationship. Secondly, there is a strong negative correlation between the ARG and ORN pools. Since there is a positive metabolic contribution of ARG, via arginase,

to the ORN pool, this negative correlation must be due to the effector role of ARG through deinhibition and/or derepression of enzyme(s) in the proximal part. The effector contribution of ARG to the ORN pool must obviously be greater than the metabolic one. The exact form of these two correlations and their interpretation will be discussed towards the end of this section.

(d) Some variation in the levels of GLU is apparent both within and between sets of flasks but there does not appear to be any consistent correlation with any of the other measured variables or determined parameters in the experimental system.

(e) No strong correlations are observed between PRO levels and other variables or parameters although, in view of their metabolic relationship, some form of positive correlation with ORN might be expected and indeed is indicated.

(f) The levels of GLY and LYS show no consistent directional variation either between or within sets of flasks. However, the relatively high levels observed in certain cultures when viewed in conjunction with the harvest yields may be indicative of a possible correlate with deviation from exponential growth.

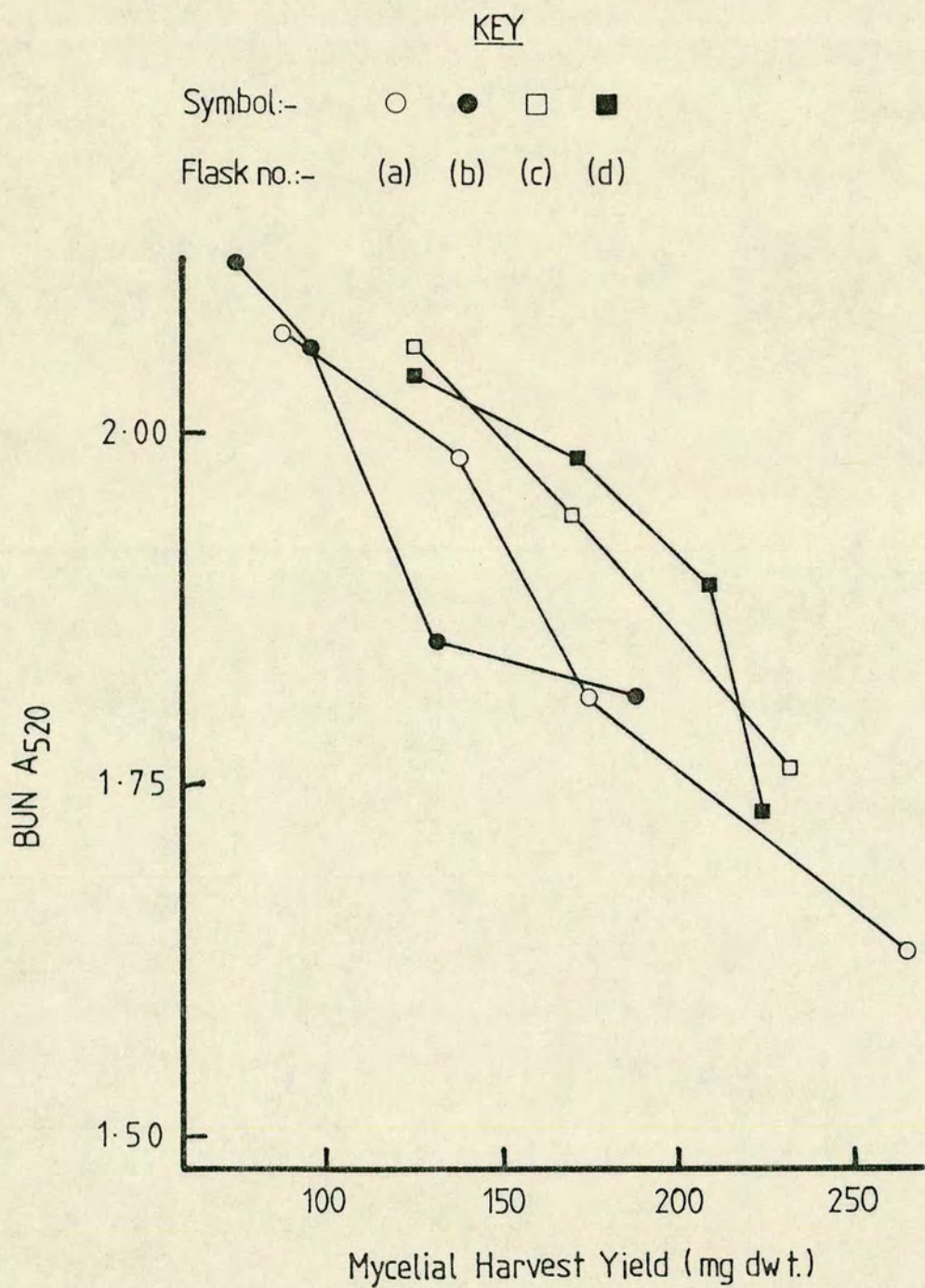
(iii) The rate of depletion of CIT from the medium

A check of the efficacy of controlling the rate of CIT uptake by varying the external HIS concentration is to measure the rate of CIT depletion from the medium. This rate should decrease with increase in HIS:CIT ratio. A convenient method for determining the rates is to assay aliquots of medium (taken immediately before harvesting each flask) by the BUN colour reaction (Section 2.2.8.1). The results of such an analysis are given in Table 7 and plotted against the corres-

Figure 12: Experiment VIa: the rate of medium BUN depletion.

Data from Table 7, Appendix III.

See accompanying text for details.



ponding harvest yields in Figure 12. Contrary to expectations the rate of depletion at different HIS inhibitions appear to be approximately the same. This anomolous result can be explained, at least in part, by the realization that urea, which is also BUN positive, would be present in certain amounts in each of the medium samples. In arg-12 ure, urea is a terminal end-product of the arginine pathway which is excreted into the medium where it accumulates. The rate of urea accumulation is proportional to the mycelial ARG pool size (Flint, 1977). The net effect of the urea accumulations would be to underestimate the true CIT depletion rate. The effect would be greatest in cultures with lowest HIS:CIT ratios (and highest mycelial ARG pools) and least in cultures with highest HIS:CIT ratios (and lowest mycelial ARG pools).

(In view of the effect of the urea accumulations it was decided that a chromatographic separation of CIT and urea was essential prior to determination by the BUN method and this was done in all subsequent experiments).

Although the contribution of urea to the total BUN positive substance in each sample would be relatively small compared with the CIT contribution this effect does preclude a rigorous interpretation of the BUN-depletion data. Nevertheless, there is no evidence for a depressed, potentially growth limiting rate of CIT depletion from the medium by those cultures supplemented with the higher ratios of HIS:CIT in which a significant departure from exponential growth was observed. It can only be presumed that the growth effect must be an indirect effect of HIS resulting from the inhibition of the translocation of ARG across the vesicular membrane, as postulated earlier (Section 4.1.6).

In conclusion, the data from this experiment indicate the potential efficacy of the methodological strategy previously outlined. The range of HIS:CIT ratios did in fact produce CIT and ARG values straddling the values characteristic of minimally grown wild-type. However, it was desirable that a wider range of ARG and metabolically related pool concentrations be generated. Furthermore, the significant growth rate effects observed at high concentrations of HIS (and ratios of HIS:CIT) in the medium were of considerable concern; unless cultures could be grown under exponential, steady state conditions the valid interpretation of results, would be seriously impaired.

4.2.2. Experiment VIb: Mycelial harvest yields and amino acid pools in ure.

This experiment was conducted in parallel with Experiment VIa as a control for comparison of the effect of HIS on the prototrophic strain, ure. The experimental design is outlined in Table 8. The salient features of the experimental data, as presented in the same table, will be discussed briefly.

(i) Growth rate

Taking into account the original conidial inoculi, the harvest yields at equivalent time elapsed since inoculation are almost identical under all growth conditions; there is no evidence for an effect of HIS, either direct or indirect, on the growth of ure under the particular conditions employed.

(ii) Mycelial aminoacid pools

Although not extensive, the data on the mycelial aminoacid pools merit comment. Firstly, the relationship between the exogenous HIS:CIT ratio and the mycelial CIT and ARG pool levels in ure agrees with that observed in Experiment VIa for arg-12 ure. Secondly,

the inverse relationship between the ARG pool levels and the level of ORN is again in evidence. The minor variations observed amongst the other pools while of undoubted interest to some are outwith the scope of discussion here. However, the general comments made with respect to the corresponding data from Experiment VIa (Table 7) apply equally.

In the cultures grown solely on CIT supplemented medium the endogenously formed HIS pool is of the order of $1 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ On supplementation of the medium with HIS the intramycelial HIS pool is expanded 30 to 40-fold.

One final observation of interest is the ARG pool values observed in cultures grown on medium supplemented with $2.5 \mu\text{M}$ HIS alone. Wild-type or ure cultures grown on minimal medium have a characteristic ARG pool of about $2 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ The two-fold elevation of the ARG pool observed in the cultures grown on HIS alone is therefore surprising. The effect appears to be specific to ARG since no other pools measured appear to be so elevated. The only ad hoc explanation which can be forwarded for this behaviour again relies on the inhibition of ARG translocation across the vesicular membrane (Section 4.1.6). In this case it must be assumed that the inhibition of ARG egress from the vesicle is greater than the inhibition of ARG uptake into the vesicle with the net effect being an expanded total extractable pool.

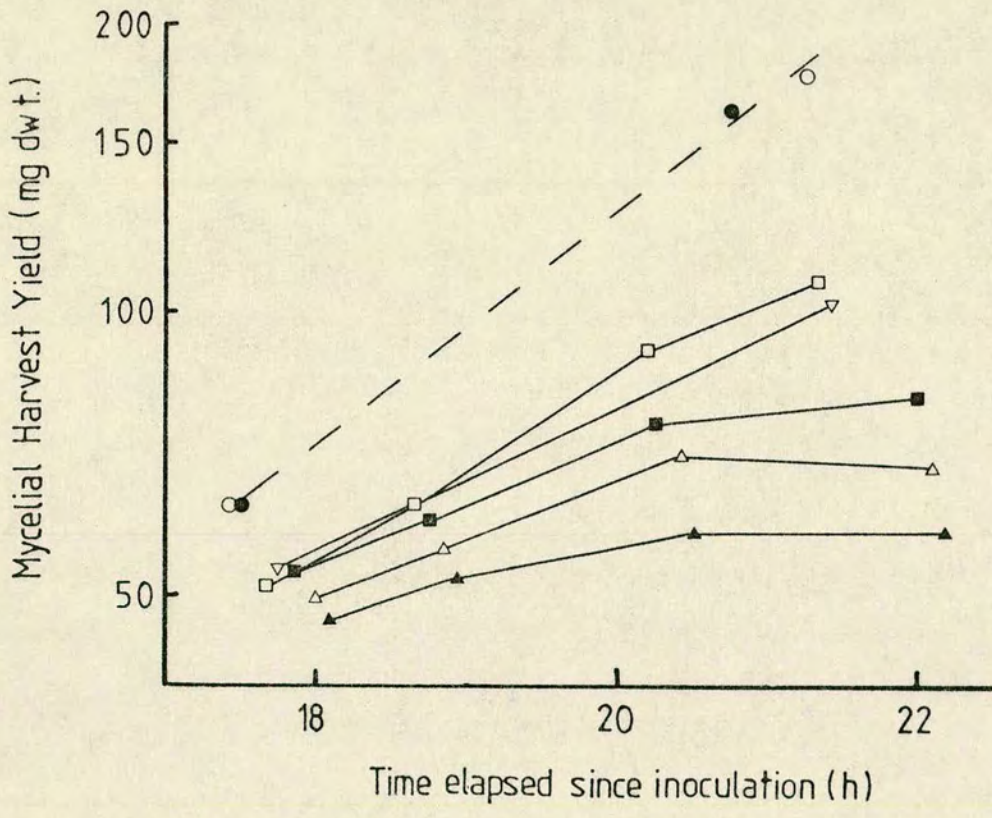
Figure 13: Experiment VIIa: growth rate.

Data from Table 9, Appendix III.

See accompanying text for details.

KEY

Symbol :-	○	●	□	▽	■	△	▲
Flask no.:-	(a)	(b)	(c)	(d)	(e)	(f)	(g)



4.2.3

Experiment VIIa: Mycelial harvest yields, aminoacid pools and enzyme specific activities in arg-12 ure

It was desirable to establish whether the range of ARG pools generated by the HIS inhibition method in Experiment VIa could be extended. The following experiment was carried out in essentially the same manner as before but with the range of HIS:CIT ratios extended. In addition, the amount of HIS added was held constant with the HIS:CIT ratio varied by varying the initial concentration of CIT. The design of the experiment is outlined in Table 9 along with the results of the various analyses performed. The growth rates for different sets of cultures were determined as were the rates of depletion of CIT from the medium and the mycelial aminoacid pools. In addition, the specific activities of enzymes involved in arginine metabolism were determined.

The salient features of the results are as follows:

(i) Growth rate

From the log plot of dry weight against time elapsed since inoculation (Figure 13) it is clear that the HIS growth rate effect is not equalised by maintaining the concentration of added HIS constant but in fact the effect increases progressively with decreasing CIT concentration. This HIS:CIT ratio-dependent reduction in growth rate with prolonged incubation in the presence of HIS provides evidence for the hypothesis, expounded in Section 4.1.6, that ARG egress from the vesicle is inhibited by HIS. Thus, despite a relatively high total extractable ARG pool, the pool of ARG available for protein synthesis can become growth rate limiting with time.

(ii) Mycelial aminoacid pools

In agreement with the results of Experiment VIa the mycelial CIT, and consequently ARG, pools are found to be directly related to the

external HIS:CIT ratio; the higher the ratio the lower the pool(s). In this experiment the range of mycelial CIT and ARG pools was extended to well below those previously observed. In addition, the pools attained on medium supplemented with CIT only are added to the range previously observed. Not only are differences in CIT and ARG pools observed between sets of cultures grown on different HIS:CIT ratios but within each set a consistent directional change in the CIT and ARG pools is also observed with successive harvests; the greater the time elapsed since HIS addition the lower are the mycelial CIT and ARG pools.

The two previous experiments indicated an inverse relationship between the ARG and ORN pool levels. The data from the present experiment expand upon that relationship. As will be shown (Figure 24, discussed later) the relationship is inverse and non-linear; a large increase in the ORN pool is observed for a small decrease in the ARG pool at ARG values below about $2 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ This is what would be expected if the enzymes catalysing steps in the pathway prior to the formation of ORN were subject to co-ordinate derepression by ARG, as has already been shown to be the case for the last three steps (Barthelmess et al., 1974). The discussion of the mechanism underlying the relationship between ARG and ORN will be returned to in Section 4.2.6.

Data from this experiment substantiate the positive correlation between PRO and ORN suggested by data from previous experiments. This could be explained by a simple mass action effect with respect to the flux from ORN to PRO through OTAase activity or may reflect an additional regulatory effect on this enzyme activity (for example, through the induction or activation of activity by ORN or deinhibition or derepression of activity by ARG). This question will be returned to after the enzyme

data have been described.

The HIS pools are generally high (30 to 40 $\mu\text{mol.100 mg}^{-1}$ d.wt.) in cultures grown in medium supplemented with HIS. However, the HIS pools are clearly dropping with time in cultures grown at the higher HIS:CIT ratios. The reason for this is unknown but it is unlikely to be due to exhaustion of the HIS from the medium. More likely it is a consequence of the effect of HIS itself on growth which causes the rate of HIS uptake to fall.

There appears to be a small but significant decrease in the GLU pool with increasing HIS:CIT ratio and with the time elapsed since HIS addition, i.e. concomitant with the growth rate effects. Under the same conditions similar effects on the GLY pool are observed but resulting with the pool increasing rather than decreasing. This effect is even more pronounced with LYS. While of undoubted interest to some these effects on GLU, GLY and LYS will not be discussed further in this text. Suffice it to say that on the evidence presented so far these pools serve as good indicators of the general metabolic state of the cultures; gross changes in their levels may be taken as indicative of a significant change in the overall metabolic state.

(iii) Enzyme Specific Activity

The biosynthetic enzyme AOGTase and the catabolic enzymes OTAase and arginase were assayed in mycelial extract supernatants as described in the Materials and Methods. AOGTase catalyses the formation of AcGLU and ORN from AcORN while OTAase catalyses the transamination of ORN to form GSA which can in turn be reduced to form PRO. Arginase catalyses the hydrolysis of ARG to ORN plus urea (see Figure 1).

Figure 14: Experiment VIIa: the relation between ARG and
OTAase.

Data from Table 9, Appendix III.

See accompanying text for details.

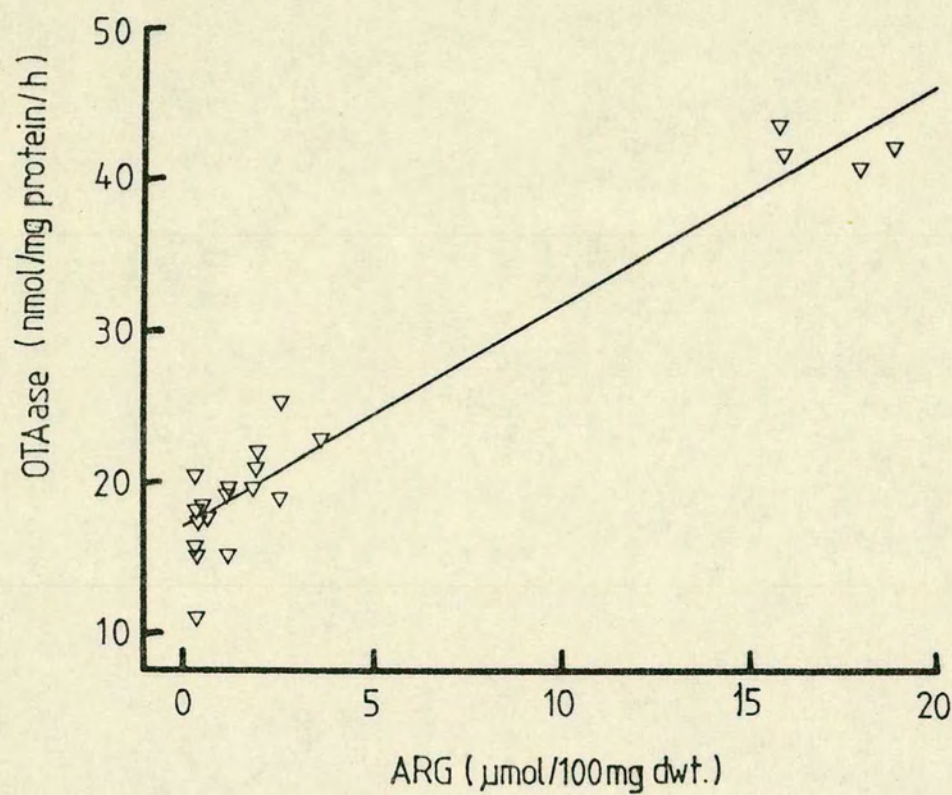


Figure 15: Experiment VIIa: the relation between ARG and arginase.

Data from Table 9, Appendix III.
See accompanying text for details.

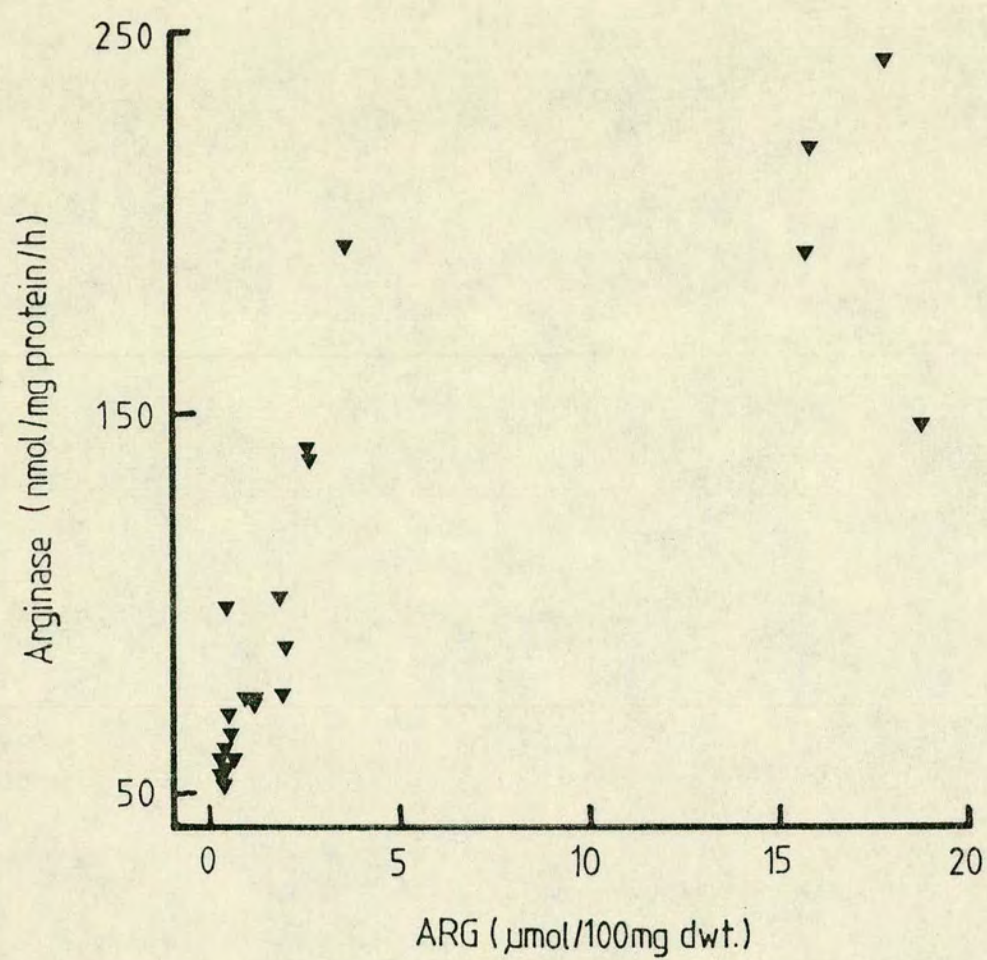
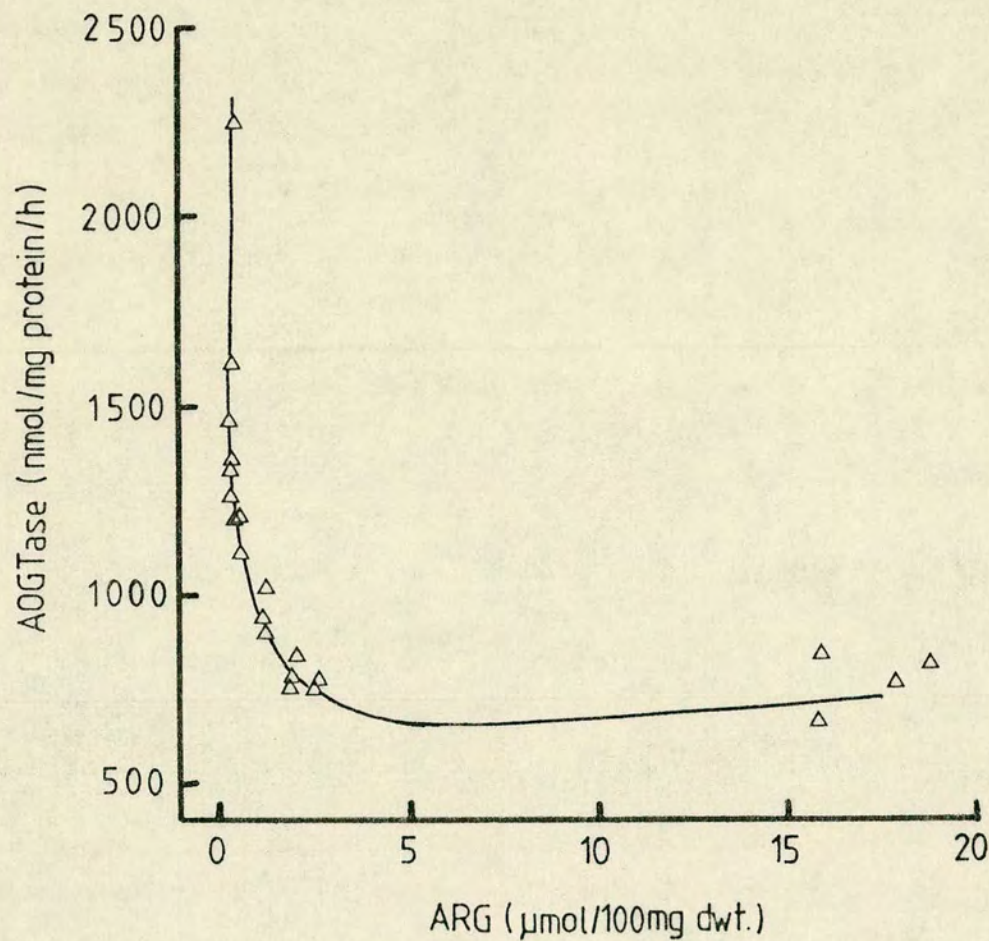


Figure 16: Experiment VIIa: the relation between ARG and AOGTase.

Data from Table 9, Appendix III.

See accompanying text for details.



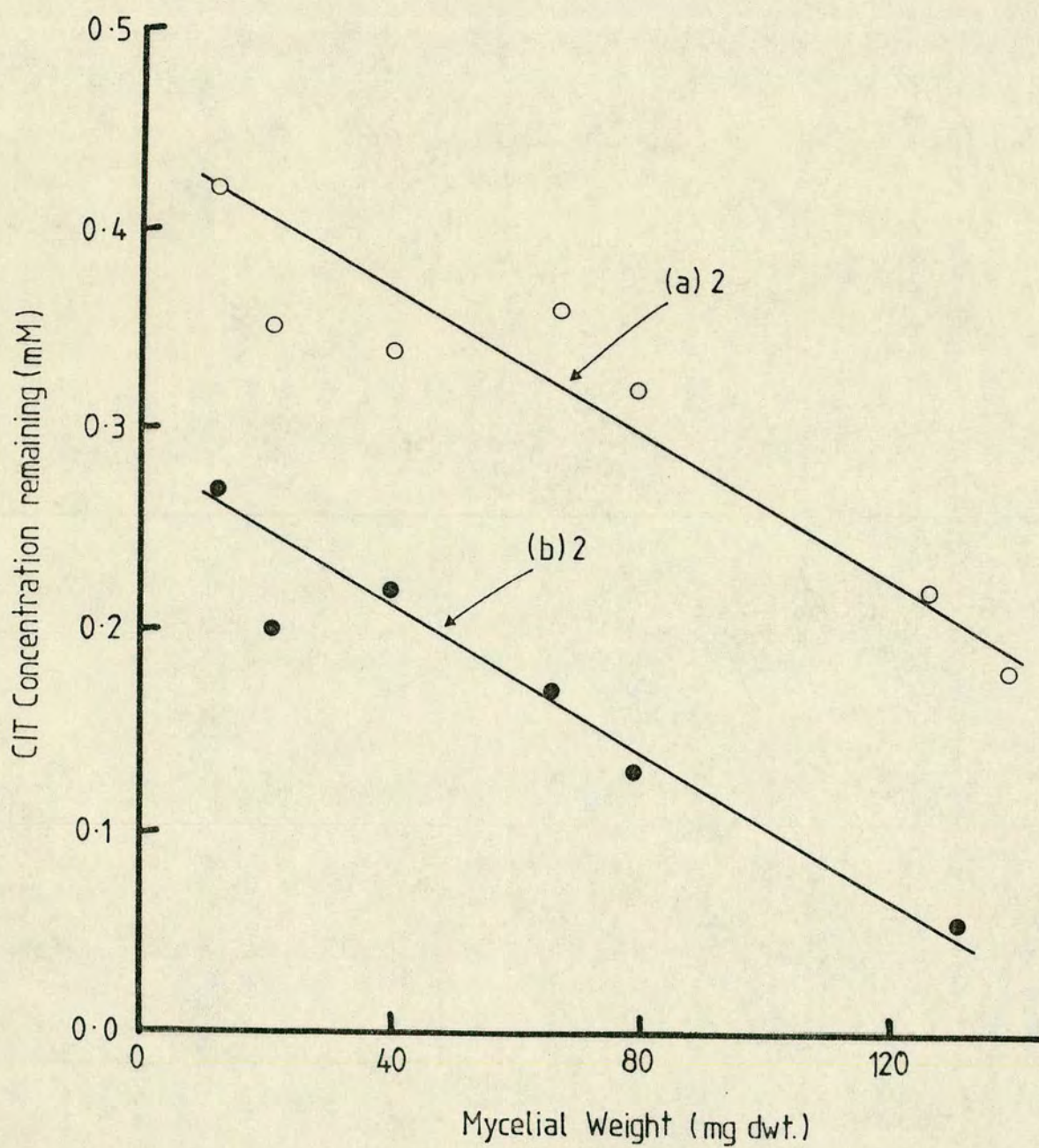
Each of these enzymes show significant changes in specific activity within and between sets of cultures. The specific activities of the two catabolic enzymes OTAase (Figure 14) and arginase (Figure 15) show a strong positive correlation with the level of the ARG pool. The experimental data are in both cases substantially clustered around the two extremes of ARG pool values and enzyme specific activities; the full form of the relationship between the ARG pool and the catabolic enzyme activities is not revealed by this data but will appear in a later section.

Nevertheless the present data are wholly consistent with ARG or some directly related pool acting as a signal for the induction of the synthesis of these two enzymes. The effect of the ARG pool on OTAase activity must be, if anything, in opposition to the observed positive relationship between the ORN and PRO pools: this inevitably leads to the conclusion that the latter relationship is the consequence of the greater importance of mass action by ORN than the catalysis between the pools.

The specific activity of the biosynthetic enzyme AOGTase changes in a quite different manner from the catabolic enzymes and in fact shows a strong negative correlation with the ARG pool. Very little change in the AOGTase specific activity is observed until the ARG pool falls to values below $2 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ after which a sharp increase in specific activity is observed (Figure 16). This relationship suggests that AOGTase may be included in the same derepression system as the last three biosynthetic enzymes of the pathway, the signal for which is ARG (Barthelmess *et al.*, 1974).

Figure 17: Experiment VIIa: the rate of medium CIT depletion.

Data from Table 9, Appendix III. See accompanying text for details.



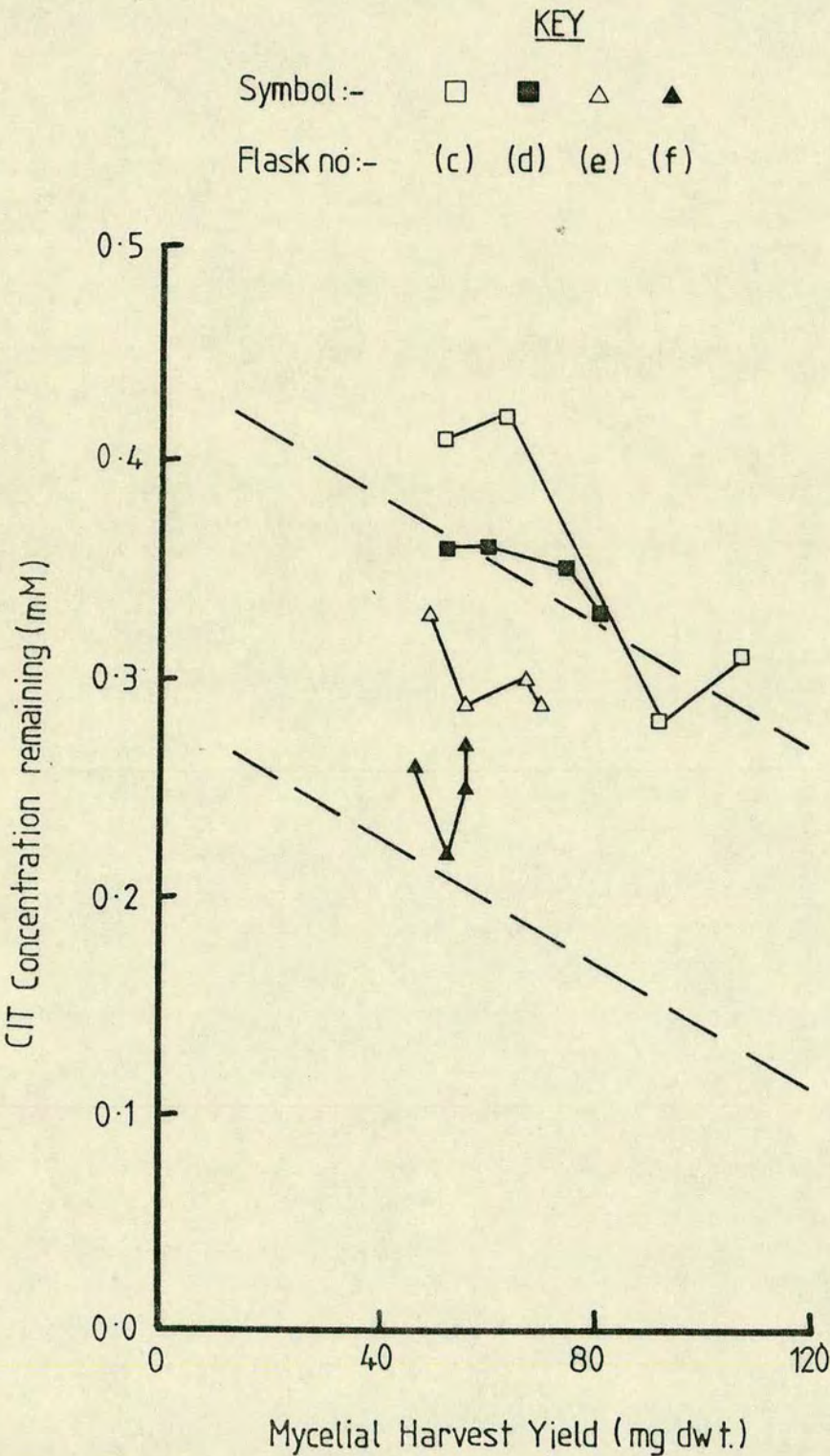
(ii) The rates of depletion of L-citrulline from the medium.

Four cultures were grown in medium supplemented with CIT alone, two containing an initial CIT concentration of 0.5 mM and two containing 0.3 mM CIT only (Table 9). Successive medium samples were taken from these flasks with time and the concentration of CIT remaining in the medium was determined by the automated colorimetric method described previously, but in contrast with Experiment VIa, CIT was first separated chromatographically from urea, as described in Section 2.2.8.1 (see Table 10 for raw data). The dry weight of the cultures at the time of medium sampling were extrapolated from the growth rate determined from the yields obtained when the flasks were harvested (Figure 13).

The concentrations of medium CIT are plotted against dry weight in Figure 17. It shows that the rate of CIT depletions were not significantly different between cultures inoculated into 0.5 mM or 0.3 mM CIT. In both cases the rate of CIT depletion was approximately 0.14 $\mu\text{mol. per ml per 100 mg d.wt. increase}$. Since the culture volumes were 400 ml this is equivalent to 56 $\mu\text{mol. per 100 mg d.wt. increase}$. The equality of depletion rates suggests that, at concentrations of 0.3 mM or above, the CIT transport system is near saturation. This contention is substantiated by the observed close similarity in mycelial aminoacid pool levels ^{at both levels} of CIT supplementation. However the slightly lower CIT (and ARG) pool levels observed in cultures inoculated into 0.3 mM CIT compared with those in cultures inoculated into 0.5 mM CIT indicates that the CIT transport K_m is not an order of magnitude smaller than the lower level of

Figure 18: Experiment VIIa: the effect of HIS on the rate of medium CIT depletion.

Data from Table 9, Appendix III. See accompanying text for details.



supplementation. Furthermore the CIT (and ARG) pools are lower under both supplementation conditions at the second compared with the first harvest over which time the medium CIT concentration fell from approximately 0.27 to 0.06 mM and 0.42 to 0.18 mM respectively. Nevertheless, for this 2 to 5-fold change in the external CIT concentration the internal CIT concentration changed by less than 30%.

That CIT is actively taken up into the mycelium is clearly demonstrated by the following calculation: the wet weight of Neurospora mycelium is approximately 2.5 times the dry weight (Slayman and Tatum, 1964). In other words, the cell water is 1.5 times the dry weight. The mean CIT pool value for the four cultures grown on CIT alone was $16 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ Therefore the minimum concentration of CIT in the cell water would have been approximately 100 mM. This means that the intermycelial CIT concentration is at least 200-fold higher than the exogenous CIT concentration under the conditions of CIT supplementation.

The data (Table 9) on the concentrations of CIT in the medium in cultures grown on various ratios of HIS:CIT are shown (together with those for cultures grown on medium supplemented with CIT alone) plotted against dry weight in Figure 18. Because of the growth rate effects of HIS, the data for cultures grown on varying ratios of HIS:CIT do not cover a great change of dry weights and consequently only a small change in medium CIT concentrations. A progressive reduction in the rate of depletion of CIT from the medium is however indicated with increasing ratio of HIS:CIT.

In summary, a range of ARG pool levels, extending above and below that observed in wild- type Neurospora grown on minimal medium,

was successfully established under conditions of exponential growth by the chosen regimes of HIS and CIT supplementation. The mycelial levels of ARG were closely related to the exogenous HIS:CIT ratio. A general decline in the ARG pool was also observed as a function of the time elapsed since HIS addition. Limits as to the efficacy of the experimental methodology were, however, indicated by the slow growth of cultures at the higher HIS:CIT ratios and the concomitant effects on the levels of aminoacid pools not directly related to arginine biosynthesis.

The observed variation in the specific activity of each of the three arginine pathway enzymes monitored is consistent with the activity of all three being controlled by the ARG pool level albeit by different mechanisms.

4.2.4

Experiment VIIb: Enzyme specific activities in ure

This experiment with the ure strain was conducted as a control for comparison with Experiment VIIa where the arg-12 ure strain was employed. The experimental design and results of analyses performed are detailed in Table 11.

Only minor differences, not justifying further comment, are observed between the growth and aminoacid pool behaviour observed here and that already described in Experiment VIb. The only notable addition is that the regime of pools characteristic of the minimally grown condition is added to the range of steady states described in ure.

However, the effect of the supplementation conditions on the activity of the four ARG pathway enzymes assayed merit brief comment.

The activity of a second biosynthetic enzyme, ASAase, was determined in addition to those in Experiment VIIb.

The activities of the catabolic enzymes OTAase and arginase show the same positive relationship with the ARG pool level as was observed in Experiment VIIa.

In this experiment, where the ARG pools are by definition greater than or equal to that characteristic of minimally grown wild-type cultures, no significant directional variation in the specific activities of the biosynthetic enzymes, AOGTase and ASAase, is observed.

Figure 19: Experiment - VIII: growth rate.

Data from Table 12, Appendix III.

See accompanying text for details.

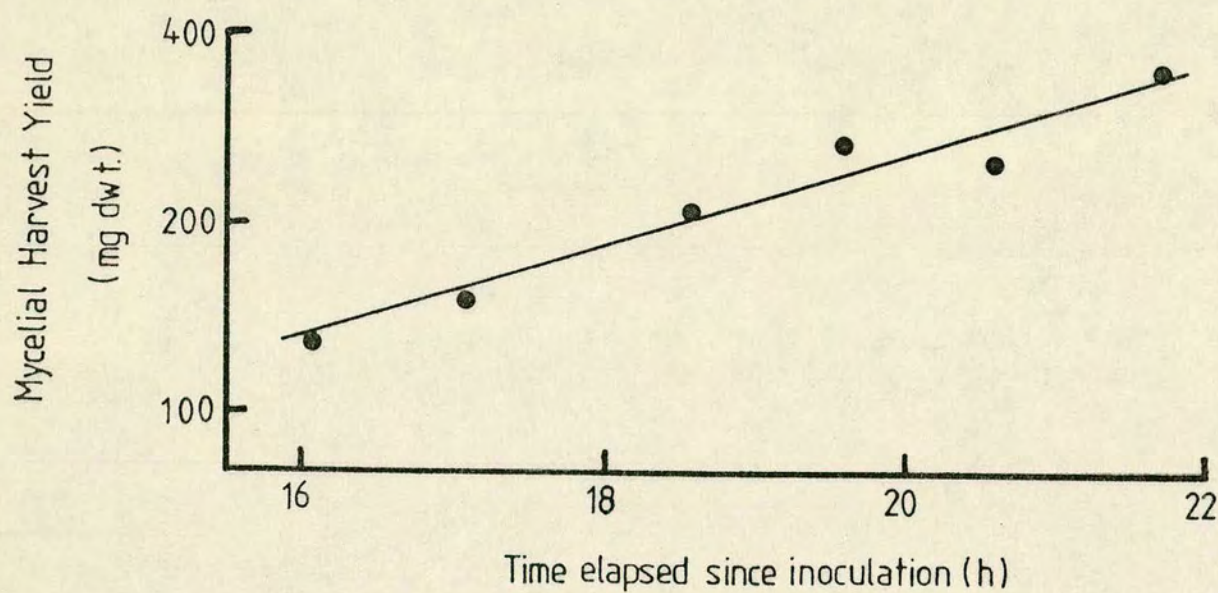
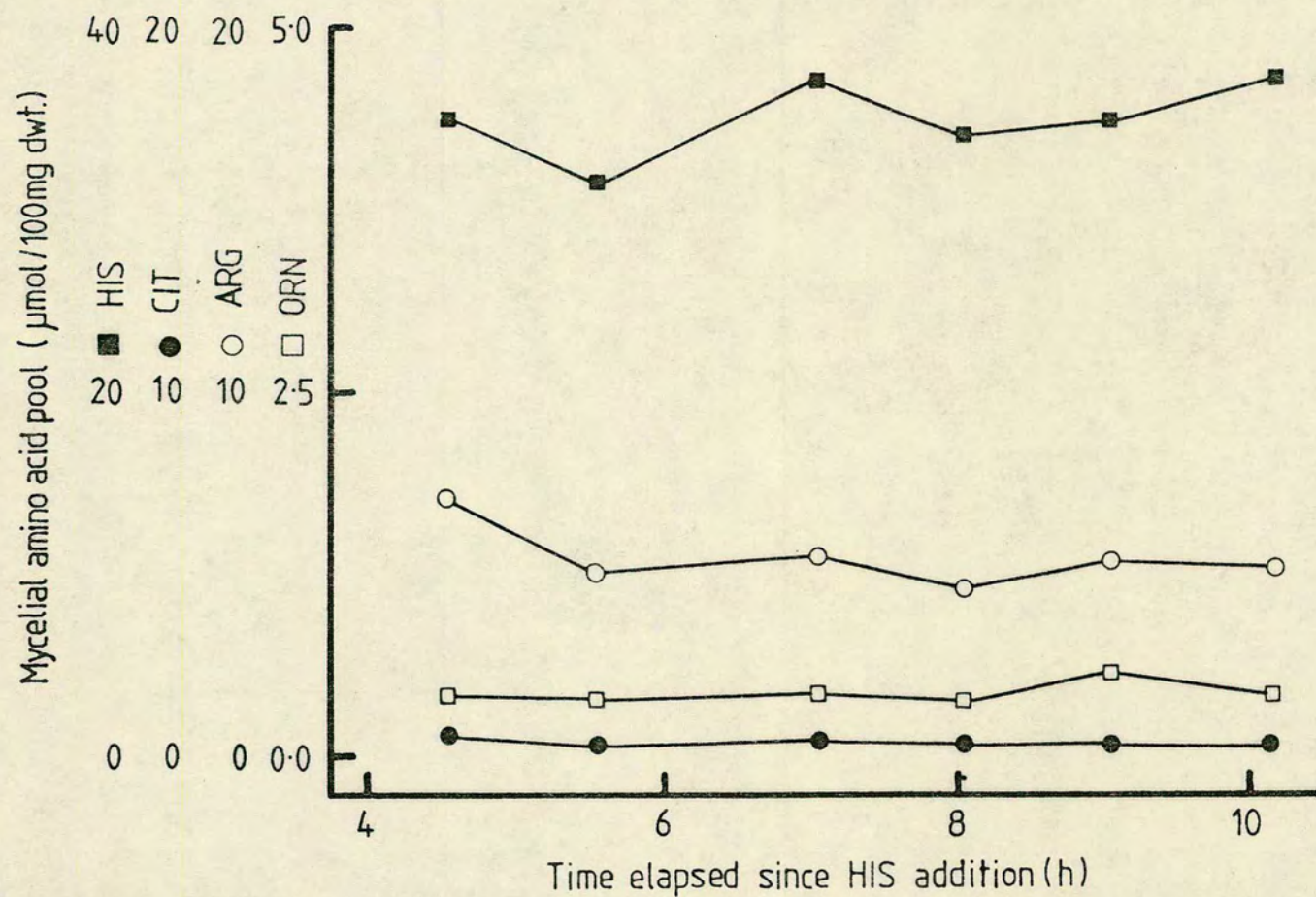


Figure 20: Experiment VIII: the time invariance of mycelial amino acid pools.

Data from Table 12, Appendix III. See accompanying text for details.



.2.5

Experiment VIII: The stability of the steady state

In each of the previous experiments several different HIS:CIT ratios were employed each determining a different metabolic steady state aminoacid pool regime but each described by only a few cultures. In the experiment to be described a more extensive analysis was conducted into the establishment of one particular, time invariant mycelial pool and enzyme milieu. The experiment was conducted in much the same manner as before; the details and results are contained in Table 12. All six growth flasks were supplemented with 0.04 mM CIT at inoculation. After 11 h 30 min incubation more CIT (0.36 mM) and, at the same time, HIS (1.20 mM) were added to each flask. After a further 4 h 35 min the cultures were harvested at successive, approximately hourly intervals as detailed in Table 12. The salient features of the experimental data are as follows:

(i) Growth rate

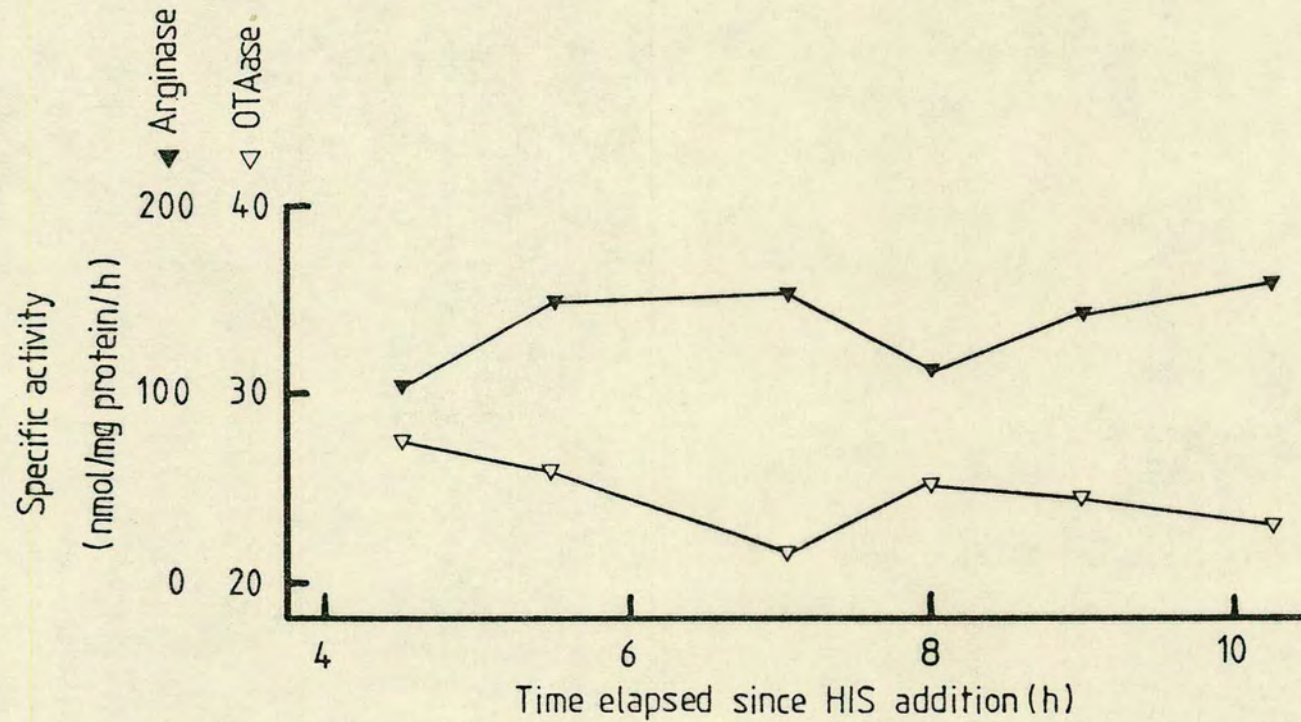
It can be seen from Figure 19 that the growth is exponential and the rate constant throughout the experimental time course. Nevertheless the doubling time 4 h 15 min is significantly longer than that characteristic of the same strain grown solely on CIT (Figure 13); this is presumably a HIS effect. However, this does not in any way impair the interpretation of the experimental data since the steady state condition depends only on exponentiality not on the magnitude of the growth rate.

(ii) Mycelial amino acid pools

In Figure 20 the mycelial HIS, CIT, ARG and ORN pools are plotted against the time elapsed since HIS addition to the medium. From the virtual invariance of each pool it can be concluded that the cultures are

Figure 21: Experiment VIII: the time invariance of enzyme specific activities.

Data from Table 12, Appendix III. See accompanying text for details.



at a constant metabolic steady state throughout the experimental time course.

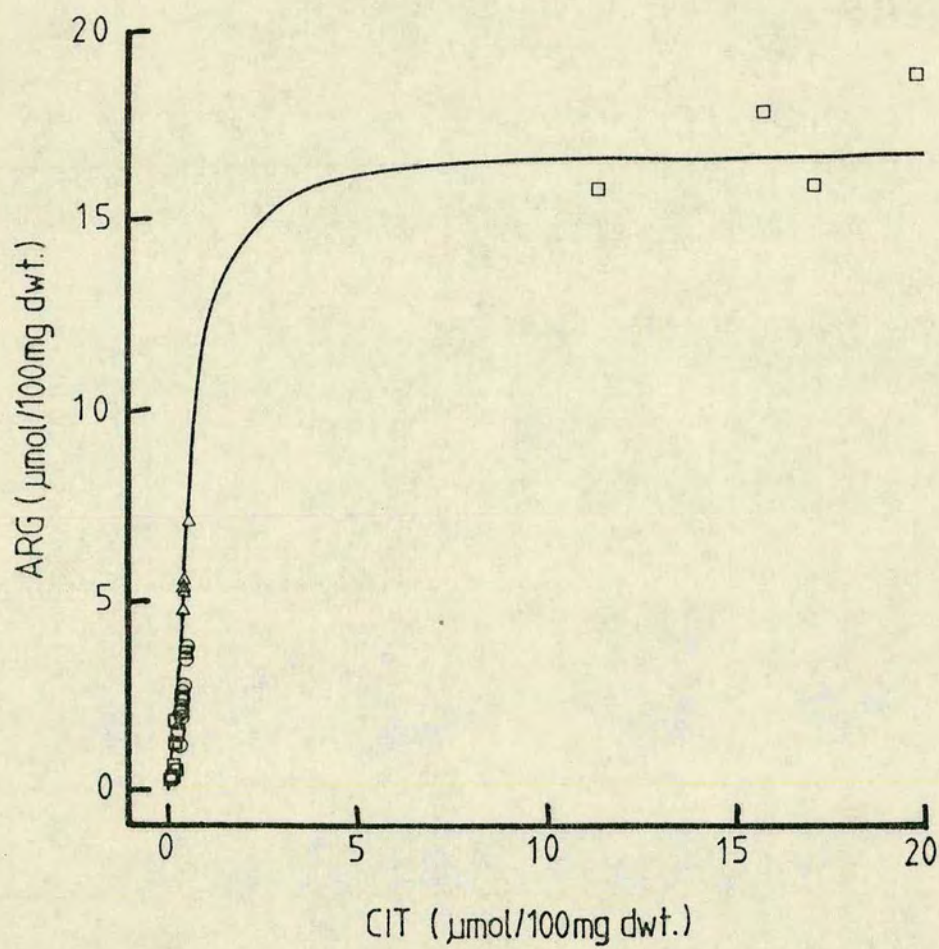
(iii) Enzyme specific activities

We have already seen that the specific activities of the AOGTase (Figure 16) and ASAase (Figure 3) are fully repressed at all ARG pools greater than about $2 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ These activities were therefore not assayed in this experiment. In contrast, the activities of the two catabolic enzymes, arginase and OTAase, appear to be modulated over the whole range of ARG pools (Figures 15 and 14). The activity of both enzymes were assayed in each culture. The results are shown in Table 12 and plotted against the time elapsed since HIS addition in Figure 21. As with the ARG pool, both activities are substantially time invariant, having values intermediate between those characteristic of CIT grown cultures and those characteristic of MIN-grown wild-type (Tables 9 and 11).

Although we have no analytical data on the past history of these cultures we can safely assume that prior to the addition of HIS the level of ARG and consequently the level of both enzymes were higher than the levels actually determined and that the expanded pool and consequently the induced activities fell with time following HIS addition to reach the values observed. If there were a significant lag between the attainment of a new signal (i.e. ARG) level and the adjustment in the level of enzyme activity then the enzyme specific activity would still be falling at the time when the ARG pool had reached its final steady state value. The experimental data (Figure 21) suggest that the beginning of the time course we have just caught the end of the pool transitions to the final steady state. Even at the beginning of the

Figure 22: The HIS:CIT steady state relation between CIT and ARG in arg-12 ure.

Data from Experiments VIa (○), VIIa (□) and VIII (△) , Tables 7,9 and 12, Appendix III. See accompanying text for details.



time course no significant change in arginase specific activity is observed, implying that the response time for the enzyme is of the same order as the rate of change in ARG. However, there is a suggestion that the OTAase specific activity is continuing to fall marginally with time indicating a slower response time. This question will be discussed more fully in the light of further data (see Section 4.3.3.2).

4.2.6 Synthesis, Summary and Conclusions

Taking together the experimental data from the five preceding experiments the following picture emerges.

- (a) HIS can be used effectively as an inhibitor of CIT uptake (Figure 18).
- (b) Depending on the ratio of exogenous HIS:CIT, the mycelial CIT pool can be controlled and the ARG pool set in arg-12 ure at steady state values between that observed on CIT supplementation alone to well below that characteristic of wild type grown on minimal medium (Tables 7, 9 and 12).
- (c) The data clearly demonstrate two important relationships between metabolically related pools. Firstly, as can be seen simply from a visual inspection of the data in Tables 7, 9 and 12, the ARG pool is positively related to the CIT pool. When plotted against each other, as shown in Figure 22, the data reveal a non-linear relationship between CIT and ARG pools. The plateau in ARG pool values beyond a certain CIT pool value strongly suggests that there is a saturation step between CIT and ARG in the pathway.

This question will be discussed further in the light of additional data (see Section 4.3.3.1).

Figure 23: The GLY:CIT steady state relation between CIT and ARG in arg-12 ure.

Data from Flint(1977) ,reproduced with the kind permission of the author.
See accompanying text for details.

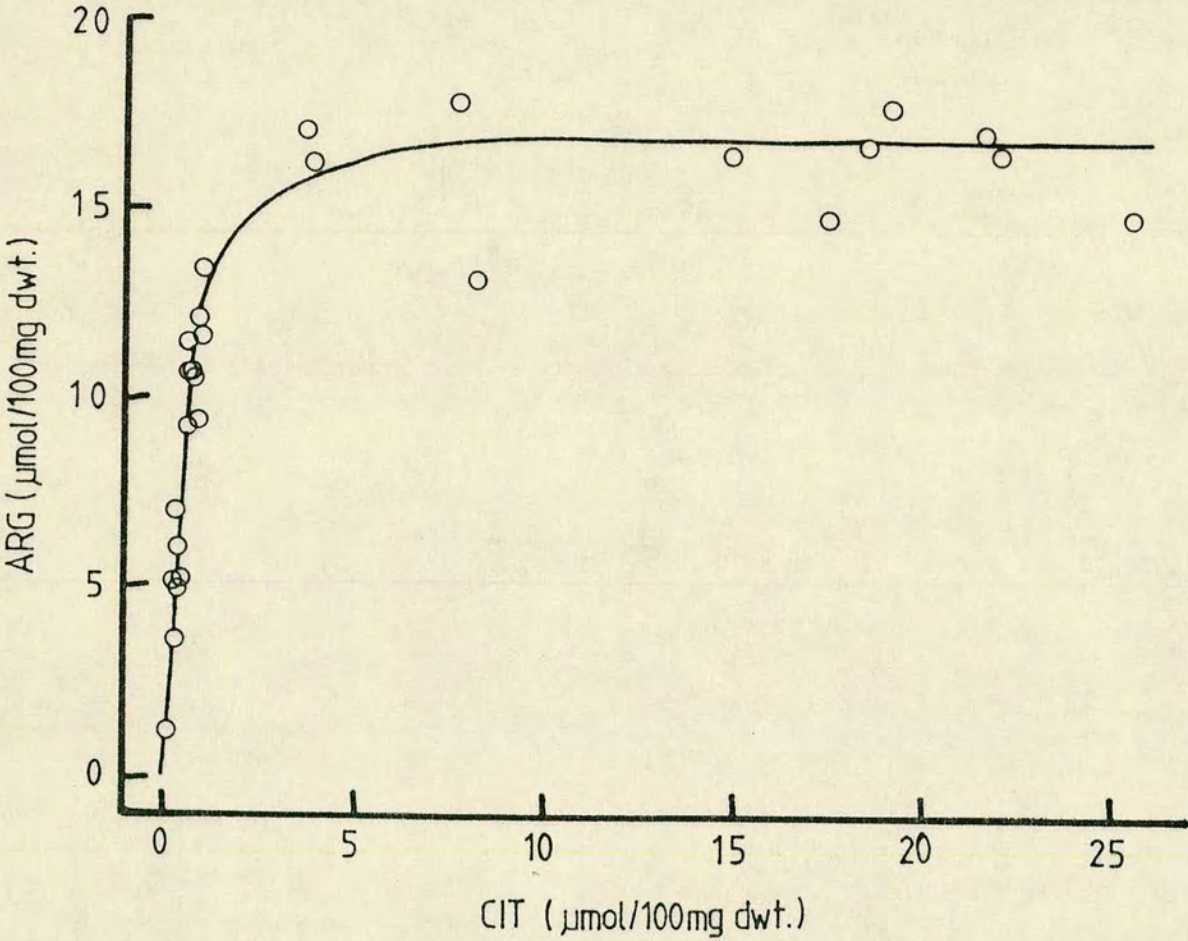


Figure 24: The HIS:CIT steady state relation between ARG and ORN in arg-12 ure.

Data from Experiments VIa (○), VIIa (□) and VIII (△) , Tables 7, 9 and 12, Appendix III. See accompanying text for details.

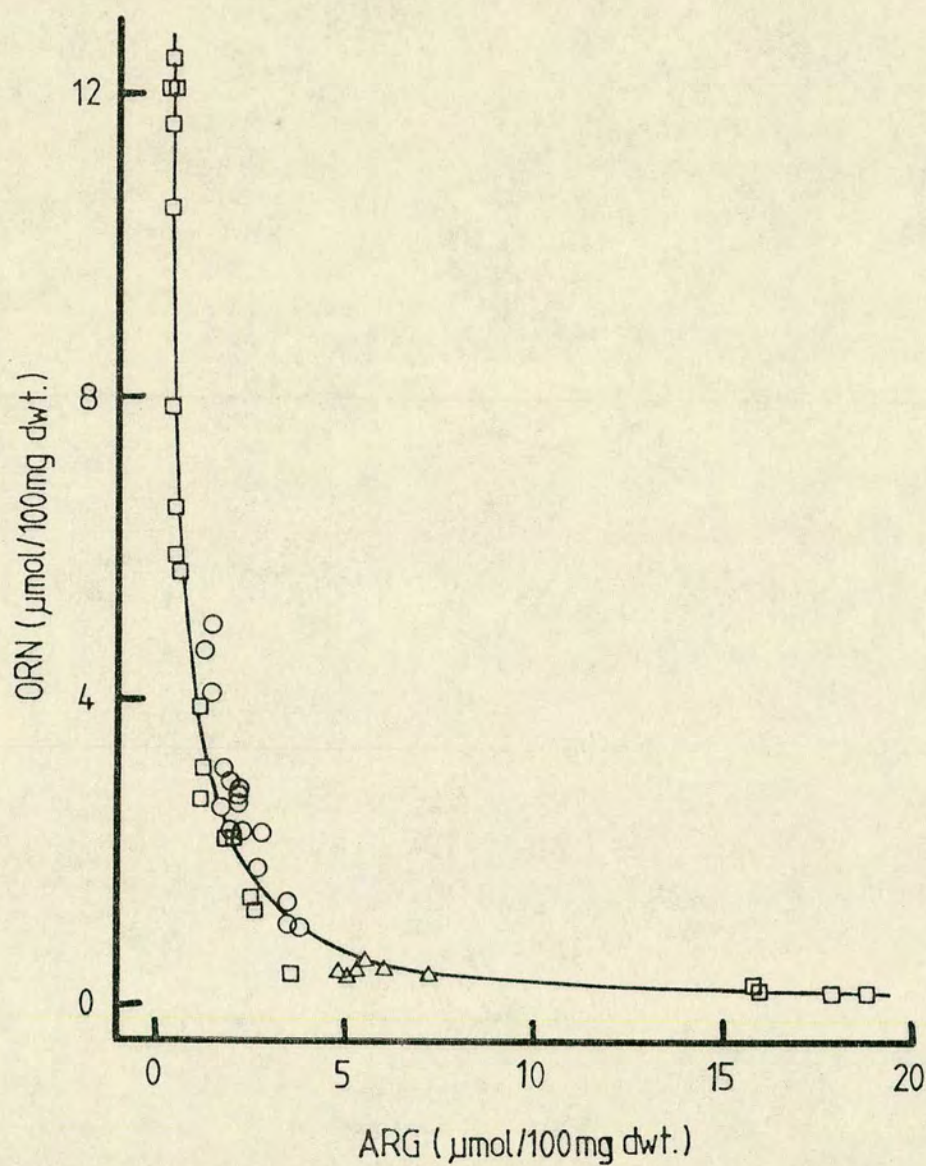
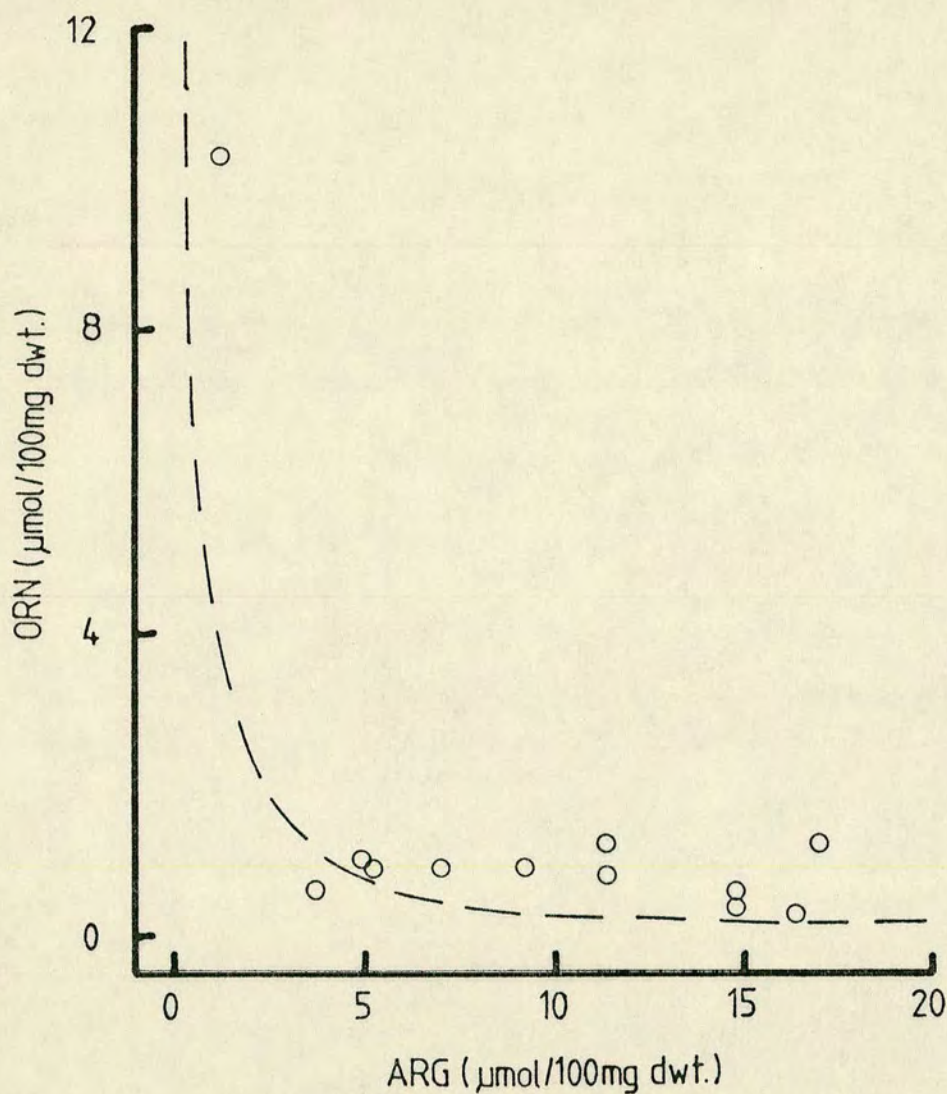


Figure 25: The GLY:CIT steady state relation between ARG and ORN in arg-12 ure.

Data from Flint(1977) ,reproduced with the kind permission of the author.
See accompanying text for details.



It should be stressed that the form of the ARG versus CIT relationship described here matches almost perfectly the same relationship described by Flint (1977) (see Figure 23). Since Flint (1977) employed GLY as opposed to HIS inhibition of CIT uptake to generate his steady state pool data this close agreement between the two studies argues strongly that the observed CIT to ARG relationship accurately reflects their true metabolic relationship and is uninfluenced by the experimental method employed to generate the steady state data.

It should be noted that the relationship drawn through the data in both Figures 22 and 23 is, in fact, the optimised plot of both the HIS:CIT and HIS:GLY data described by Flint, Porteous and Kacser (in preparation), based on a graphical treatment described by Kacser and Burns (1977).

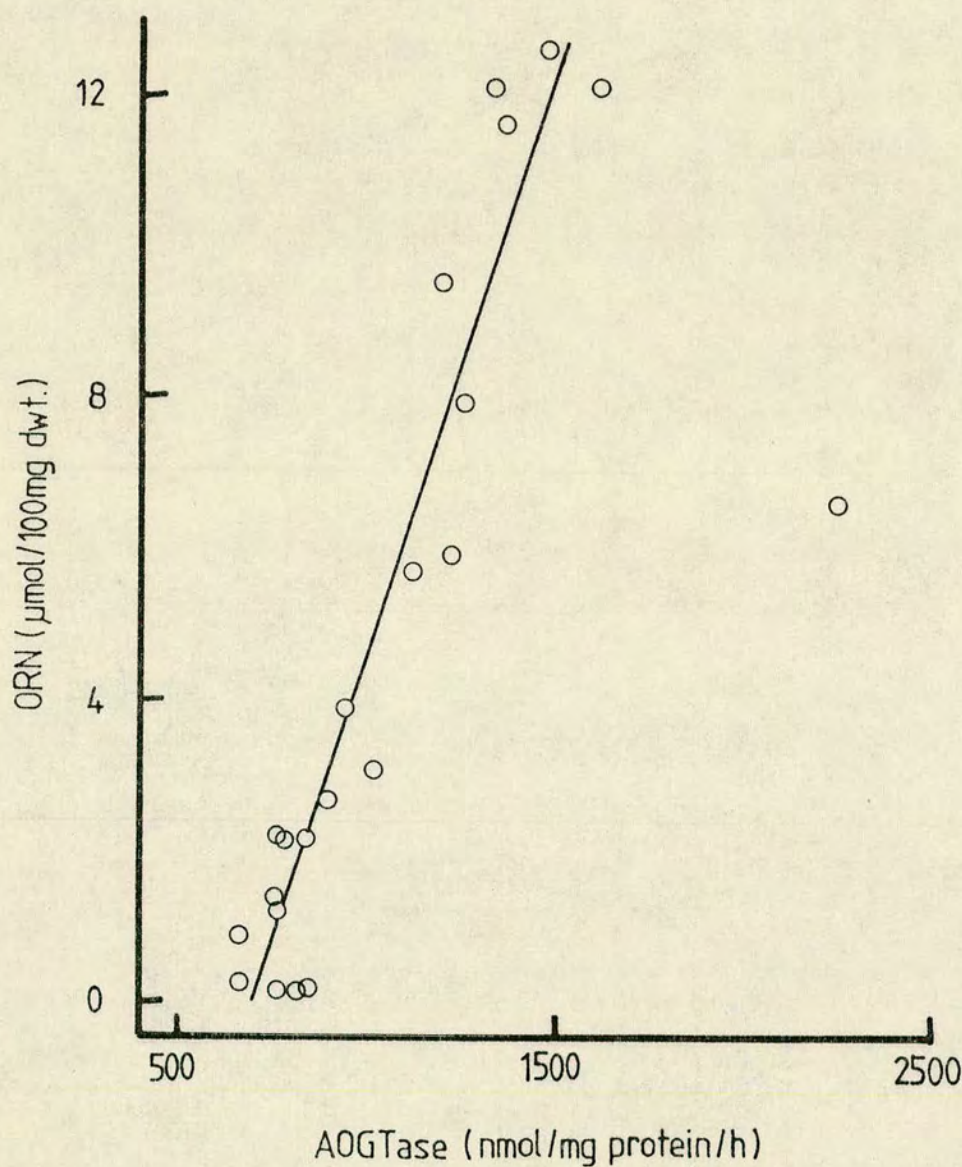
(d) Visual inspection of Tables 7, 9 and 12 indicates an inverse, non-linear relationship between ORN and ARG in arg-12 ure. The accumulated data on ARG and ORN pools are plotted against each other in Figure 24. The ORN pool increases slowly as the ARG pool falls from about 20 to 1 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ As the ARG pool falls further the ORN pool increases dramatically. Since a reduction in the ARG pool would reduce the metabolic contribution to ORN from ARG this indicates a considerable increase in flux through the early steps of the arginine pathway to ORN which must be the consequence of the effector role of ARG on the quantity and activity of the relevant enzymes.

Although not extensive, the data of Flint (1977) for these same two pools under the GLY:CIT steady state condition agree closely with the relationship just described (see Figure 25).

Figure 26: Experiment VIIa: the relation between AOGTase and ORN.

Data from Table 9, Appendix III.

See accompanying text for details.



- (e) The activities of each of the four enzymes studied appear to be affected by the ARG pool. The relationship between the ARG pool and OTAase (Figure 14) and arginase (Figure 15) activity are consistent with, though not definitive of, the suggestion that ARG or some directly related pool acts as the induction signal for both these catabolic enzymes. The correlation between the ARG pool and AOGTase activity (Figure 16) strongly suggests that this biosynthetic activity is regulated by a similar if not the same derepression system shown to regulate the activities of the enzyme catalysing the last three steps in the pathway to ARG biosynthesis (Barthelmess *et al.*, 1974).
- (f) In the arg-12 ure strain the flux through the early steps in the arginine pathway is indicated by the level of accumulated "end product", ORN.

Figure 26 shows that for about a two-fold increase in AOGTase specific activity the ORN pool increases about 25 fold. Clearly, the derepression of this and even all the other enzymes in the proximal part of the pathway cannot by itself account for this large movement of ORN. In fact, there are two additional changes whose effect is known to decrease the ORN pool and which have been shown to operate. Firstly, the ORN increase is accompanied by a decrease of some 20-fold in ARG and at least a 5-fold decrease in arginase (Figures 22 and 15, respectively). Both these changes will reduce the ARG-derived ORN considerably. The minimum decrease in metabolic contribution of ARG to ORN would be 5-fold even if arginase were fully saturated.

Secondly, the PRO values increase with increasing ORN in spite of a 2 to 3-fold decrease in OTAase activity, (Tables 7 and 9). This again indicates a change in flux out of ORN opposite to the observed increase in its concentration. This leads inevitably to the

conclusion that an additional factor increasing the flux to ORN must be operating. This factor must be the deinhibition of one or several enzymes proximal to ORN, and furthermore, the effect is likely to be an allosteric one with a high Hill coefficient. This question will be discussed in more detail later, in the light of additional data from experiments yet to be described.

(g) Limits with regards the efficacy of the experimental methodology are indicated by the effects on growth of HIS, particularly at high HIS:CIT ratios. The nature of the effects are complex. This data and the observations of previous investigators (Tateson, 1971 and Flint, 1977) suggest that at high concentrations HIS both retards germination and increases mycelial doubling time. These effects have already been discussed (Section 4.1.6). Briefly, it was concluded that medium HIS had a direct, concentration dependent, inhibitory effect on growth and development, including the appearance of aminoacid transport systems. These effects were largely obviated by delaying the addition of HIS supplement until about 12 h after inoculation.

There appears, however, to be a second independent and indirect effect of HIS seen as a decline in the exponential rate several hours after the addition of HIS supplement (Figures 11 and 13). Unlike the effect on early growth on development, this effect appears to be related to the HIS:CIT ratios, rather than the absolute HIS concentration, and hence to some metabolic consequence of CIT transport limitation. A working hypothesis (which needs to be tested by direct experimentation) has been constructed to explain this behaviour and the reversibility of HIS-mediated growth inhibition by CIT (see Section 4.1.6). Briefly, it has been shown (Weiss, 1973)

and Flint, 1977) that of the total extractable ARG pool only about 5% is cytosolic and available for protein synthesis, the rest being sequestered within a vesicle. Although these two subpools exchange fairly rapidly under steady state conditions (Flint, 1977) the observations made here can be explained if the egress of ARG from the vesicle into the cytosol is inhibitable by HIS. This is a reasonable postulate since it is known that, like ARG, most of the mycelial pool of HIS is normally held in the vesicle (Ferrier, 1978) and, in addition HIS and ARG are known to share a common transport system at least at the mycelial membrane level (Pall, 1970a). Since the rate of CIT uptake, and consequently the flux to ARG, is progressively inhibited at the mycelial membrane by increasing HIS:CIT ratios, the cytosolic ARG pool, as a result of the additional effect of HIS on the distribution of ARG, may fall to values below that necessary to maintain normal protein synthesis. This will occur despite a relatively high total extractable ARG pool remaining.

This hypothesis has been extended to say that translocation of ARG across the vesicular membrane in either direction is inhibited by HIS.

While the steady state HIS:CIT experiments just described give the basis for a full analysis of the system under study the minor deficiencies in the approach discussed above made it desirable to modify the methodology. This modification and its exploitation will be discussed immediately following the description of a final steady state experiment where novel aminoacid supplements were employed.

4.2.7

Experiment IX: the effect of medium ARG and ORN on mycelial aminoacid pools, enzyme specific activities and fluxes in ure.

In each of the previously described experiments the ARG pool has been exclusively derived from mycelial CIT via SYNase and ASAase activity. In the present experiment the consequences of supplying ARG exogenously on mycelial pools, fluxes and enzyme activities were examined. Similarly the effects of exogenously supplied ORN on these system components were also studied. The experiment was carried out as described in Table 13, wherein are contained the results of the various analyses performed. The following general observations can be made and conclusions drawn from the experimental data:

(i) Growth rate

The harvest yields at equivalent times after inoculation are essentially the same irrespective of the culture condition. There is no evidence for an effect on growth arising from the addition or omission of either of the supplements used.

(ii) Mycelial aminoacid pools

Within each culture condition each pool level is essentially constant whereas there are characteristic differences in the levels of individual pools between different culture conditions, i.e. each supplementation condition employed determines a distinct metabolic steady state. The pool data from those cultures grown in minimal medium or medium supplemented only with arginine agrees closely with those reported by other investigators (Tateson, 1971; Barthelmess et al., 1974 and Flint, 1977).

The ARG pool observed here in ARG grown cultures is significantly lower than that characteristic of CIT grown cultures

(see Tables 8 and 11).. Other workers in this laboratory have also consistently observed this phenomenon. The underlying mechanism is believed to be the feedback or trans-inhibition of ARG on its own uptake (but not on that of CIT).

Consequences of an exogenous, as opposed to solely endogenous, source of ARG are also evident in the ORN and PRO pools. Both these pools are significantly higher in ARG-grown compared with CIT-grown ure (compare Tables 11 with 13). This implies that both the arginase and OTAase fluxes are significantly increased on growth in ARG supplemented over CIT supplemented media. This conclusion appears to be contradicted by the analysis of the arginase and OTAase specific activities characteristic of these different culture conditions. It has already been shown that the specific activities of both these enzymes correlate closely with the ARG pool (Figures 14 and 15). Their specific activities are several fold higher in ARG grown cultures than MIN grown cultures but higher still in CIT grown cultures (compare Tables 11 with 13). Nevertheless this same conclusion about the different fate of exogenously and endogenously derived ARG has been arrived at by other investigators (Karlin et al, 1976; Weiss and Davis, 1977 and Flint, 1977) on the basis of various different observations. The apparently conflicting evidence from ARG pools, enzyme specific activities and fluxes under different conditions of growth can be reconciled by consideration of the subcellular organisation of the ARG pathway (Figure 2) and the kinetic characteristics of the arginase and OTAase enzymes in vitro, as previously discussed in Section 1.3.2.1. Briefly, both the ARG and ORN pools are compartmented within the mycelium with the bulk of each sequestered from the cytosol within

metabolically inert compartment, the vesicle (Weiss, 1973). The enzymes arginase and OTAase are restricted in location and in activity to the cytosol (Weiss and Davis, 1973). In minimally-grown cultures of wild-type Neurospora the cytosolic concentrations of ARG and ORN are probably less than the respective K_m 's of arginase and OTAase (Weiss and Davis, 1973 and Subramanian et al., 1973). The low flux through these enzymes in CIT-grown cultures despite a considerably expanded total ARG pool, is explained by the fact that most of this endogenously derived ARG is compartmented within the vesicle. Only when ARG is exogenously derived is it significantly hydrolysed by arginase to ORN (Weiss and Davis, 1977 and Flint, 1977). This ORN formed in the cytosol can then be transaminated by OTAase to GSA. Similarly, exogenously derived ORN is predominantly catabolised by OTAase to GSA (Davis, 1968 and Weiss and Davis, 1977).

The comparative pools for ARG and ARG plus ORN-grown cultures are also in complete accord with the foregoing summary of conclusions of previous investigators. The ORN pool is at least twice as high and the PRO pool slightly higher in cultures grown in ORN in addition to ARG. This indicates a substantial flux of exogenously supplied ORN into cytosolic ORN in the mycelium and thence to PRO via GSA. The ARG pools do not however differ significantly indicating that the endogenous flux to ARG is essentially the same between the two growth conditions. Thus exogenously derived ORN appear to be substantially sequestered from mitochondrial OTCase and channeled to cytosolic OTAase.

(iii) Enzyme specific activities

The specific activities of AOGTase and ASAase are substantially the same under all the growth conditions in accord with the

previously described relationship between these activities and the mycelial ARG pools (Figures 16 and 3).

In contrast, but as expected (Figures 14 and 15), the specific activities of OTAase and arginase vary between conditions in relation to the corresponding mycelial ARG pool. The modulation of these activities and the fluxes carried by them under different conditions have already been discussed.

It is interesting to note that the level of arginase observed here in ARG-fed cultures is of the same order expected for the same total extractable ARG pool in CIT fed cultures. This would indicate that the relationship between the signal pool and the total extractable pool is the same under both growth conditions. It has been generally assumed that the signal pool is in fact the cytosolic ARG pool, or directly derived therefrom. If true, it follows that the cytosolic to vesicular pool ratio must be the same whether ARG is endogenously or exogenously derived. Consequently, the higher arginase flux at the same pool level under ARG fed as opposed to CIT fed conditions can only be accounted for by constructing the ad hoc hypothesis that arginase is not only restricted in location to the cytosol but more specifically is closely associated with the mycelial membrane in close proximity to the point of ARG entry from the medium (Karlin et al, 1976) (The same argument would apply to OTAase to explain the observed high rate of catabolism of endogenously supplied ORN and relatively low rate of catabolism of endogenously biosynthesized ORN).

It should be added that the data of Flint (1977) suggest that on MIN medium the cytosolic ARG pool in ure is about 1% of the

total, rising from 2 to 5% of the total pool in CIT fed cultures and reading as high as 20% of the total pool in ARG fed cultures. If these estimates are assumed to accurately reflect a real difference in the cytosolic ARG pool between the ARG fed and the CIT fed condition, then the differences in arginase flux between the two conditions for the same total ARG pool can be accounted for by mass action. However, this leads to the unlikely assumption that the cytosolic pool is not the signal for arginase induction since the same relationship appears to hold between the total extractable ARG pool and the specific activity of arginase (and OPAase) irrespective of the origin of ARG. The exact form of the relationship between the ARG pool and the level of catabolic enzyme induction will be discussed in greater detail in Section 4.5.3 in the light of additional data.

4.3

The "Run Down and "Run Up" Condition : the Quasi-Steady State

In section 4.1 it was shown that for normal growth to be maintained in the arg-12 strain the addition of HIS, whatever concentration, had to be delayed by at least 10 h after inoculation into CIT supplemented medium (Tables 4 and 5). This inevitably means that the initial incubations with CIT alone will produce a very high CIT-derived ARG pool which must fall, by growth dilution and metabolism, to the final value determined by the degree of CIT uptake inhibition. The "tail end" of this temporal change is in fact evident from the quasi-steady state data presented in Section 4.2 as the tendency for CIT and ARG pools to fall with the time elapsed since HIS addition (Tables 7 and 9).

It was decided to exploit this consequence of the experimental procedure by monitoring the changes in mycelial pools and enzyme activities with time immediately following the addition of HIS. A direct comparison of such data with the previously reported steady state data would be valid if the rate of change in the CIT pool, following the sudden, drastic reduction in rate of uptake, were slow enough compared with the relaxation times of subsequent steps. In such a case the instantaneous pool values correspond to steady states with equivalent pool relations. The advantages of such a procedure are:

(i) the reported effects of increasing growth inhibition with increasing HIS concentration can be obviated in an experiment with a single concentration of added HIS and hence single degree of CIT uptake inhibition.

(ii) by continually monitoring the changes in system components with time the past history of the system is known and hence certain possibilities, which would otherwise have to be considered, may be eliminated from the interpretation of responses.

The exploitation of this method is described in the following experiments:

4.3.1

Experiment Xa: the post-HIS addition changes in mycelial amino-acid pools and enzyme specific activities in arg-12 ure.

Since the principal aim of this investigation was to generate within a single exponential growth experiment a continuum of mycelial ARG pools, from fully expanded to well within the derepression range, and to analyse the metabolic responses consequent upon such modulation it was decided, based on the results of the steady state experiments described in Section 4.2, to experiment with a 5:1 ratio of HIS:CIT in the medium. The results of such an experiment are as follows:

Fourteen growth flasks, supplemented in every case with 0.4 mM CIT were each inoculated with arg-12 ure conidia. After 17 h incubation, HIS was added to twelve of the cultures, to yield in each case a concentration of 2.0 mM. The first such culture was harvested 10 min after the addition of HIS to the medium with the subsequent cultures harvested at successive half hourly intervals. Of the two cultures to which no HIS was added, one was harvested immediately before the time of HIS addition, the other at the end of the experimental time course. The complete experimental details are contained in Table 14, along with the results of the analyses

Figure 27: Experiment Xa: growth rate.

Data from Table 14, Appendix III. See accompanying text for details.

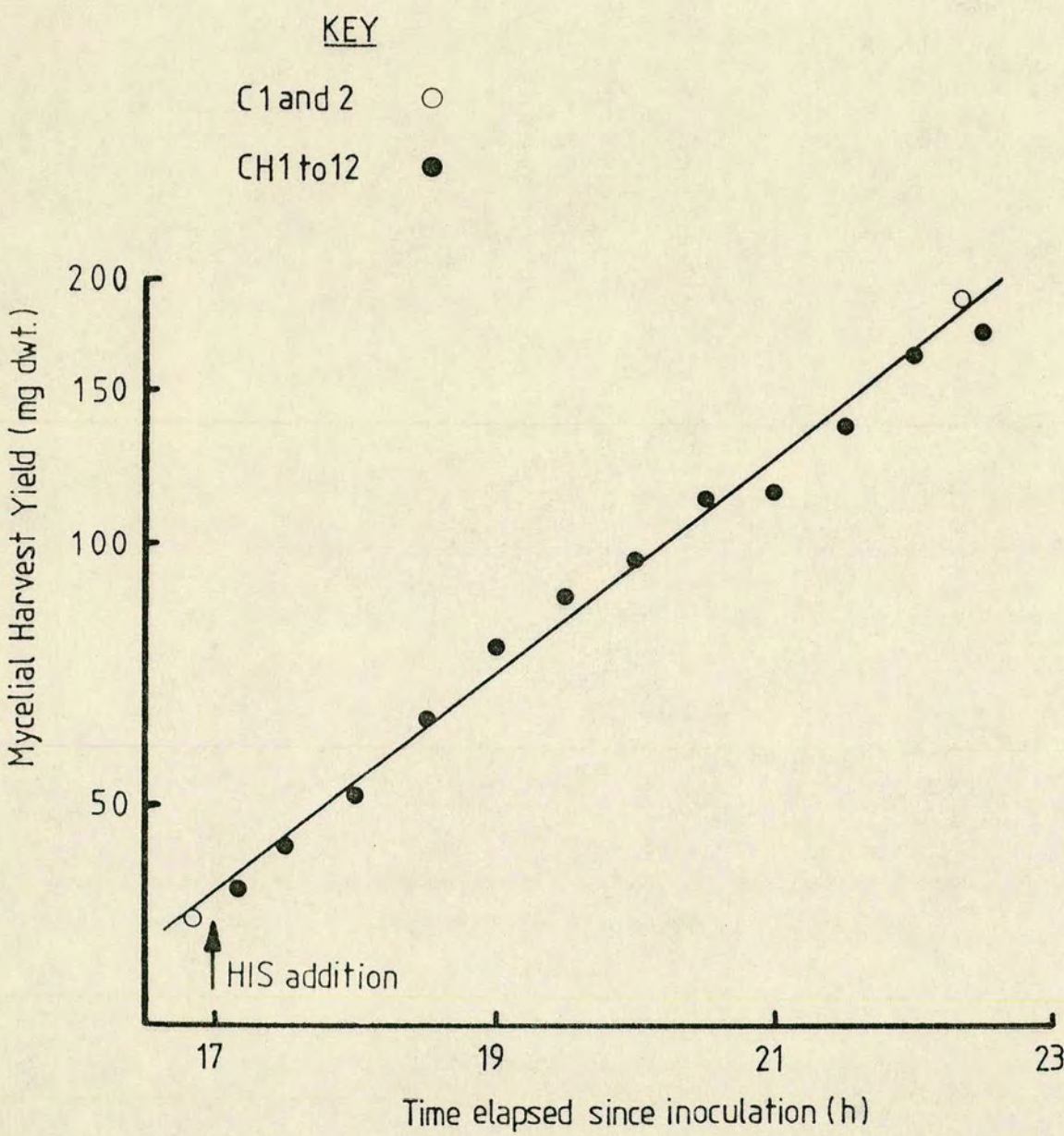
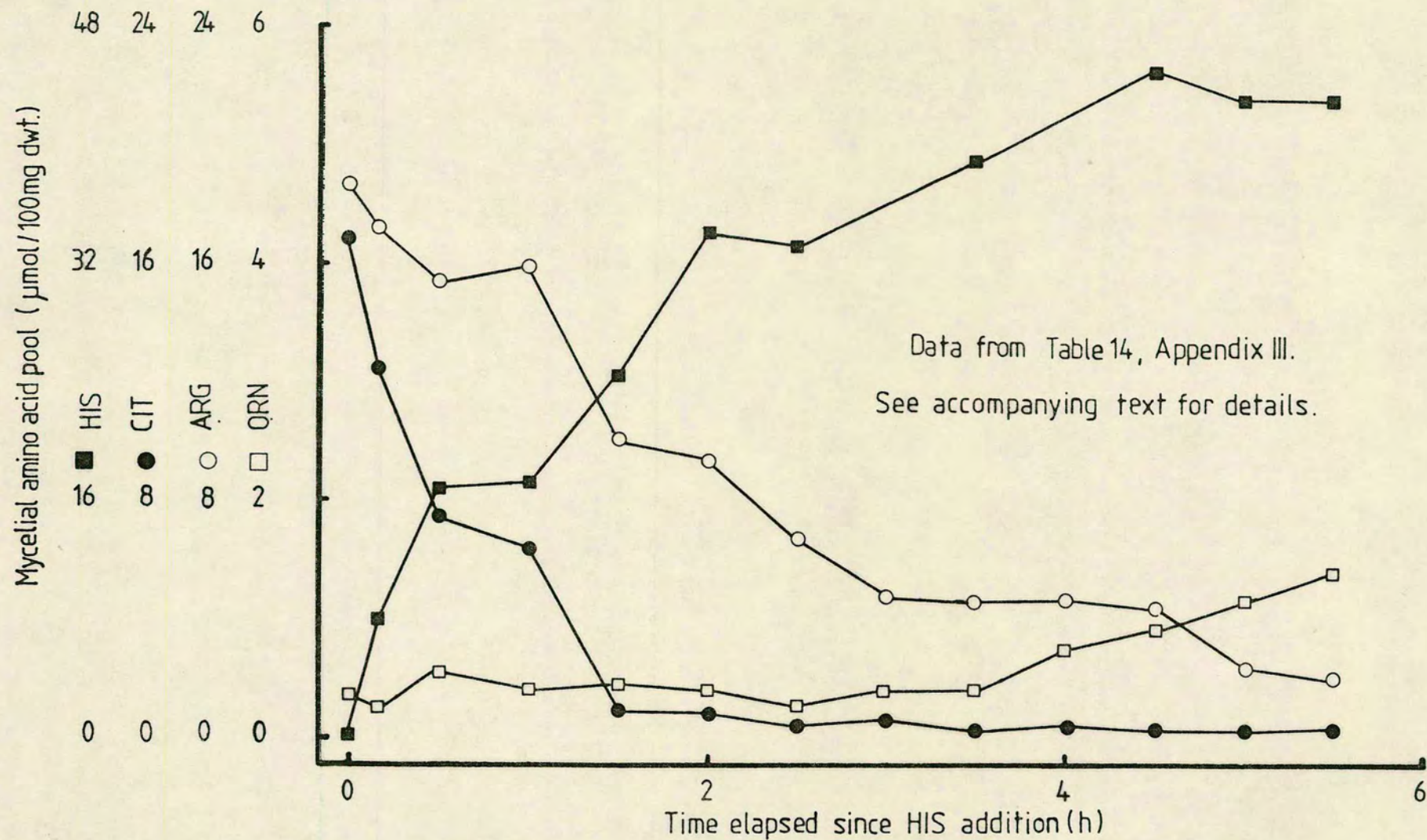


Figure 28: Experiment Xa: the post-HIS addition changes in mycelial amino acid pools.



performed on the harvested mycelia. The data permit the following general observations and conclusions to be drawn:

(i) Growth rate

The log of the dry weight mycelial harvest yields are plotted against the time elapsed since inoculation in Figure 27. The figure shows that there is no difference in the exponential growth rate of those cultures supplemented with HIS from that of the cultures grown solely on CIT. It may be concluded, therefore, that, in this particular experiment, the addition of HIS to the medium had no detectable effect on the growth of arg-12 ure cultures. It will be recalled that in Experiment VIIa (Figure 13) under identical levels of HIS:CIT supplementation a growth rate effect was observed. It must be presumed that the complete absence of effect here was due to delaying the addition of HIS from 12 until 17 h after inoculation and presumably therefore until a later developmental stage had been reached (see Figure 10).

(ii) Mycelial aminoacid pools

The mycelial HIS, CIT, ARG and ORN pool levels are plotted against the time elapsed since the addition of HIS to the medium in Figure 28 from which the following observations can be made and conclusions drawn:

a) Immediately following the addition of HIS to the medium the mycelial HIS pool expands rapidly from a basal, CIT-grown, steady state value of approximately $0.2 \mu\text{mol.100 mg}^{-1}$ d.wt. A new mycelial HIS pool steady state value of approximately $43 \mu\text{mol.100 mg}^{-1}$ d.wt. is approached after about 4 h incubation in the presence of exogenous HIS.

b) Prior to the addition of HIS to the medium, the mycelial CIT pool in CIT-grown cultures is characteristically high.

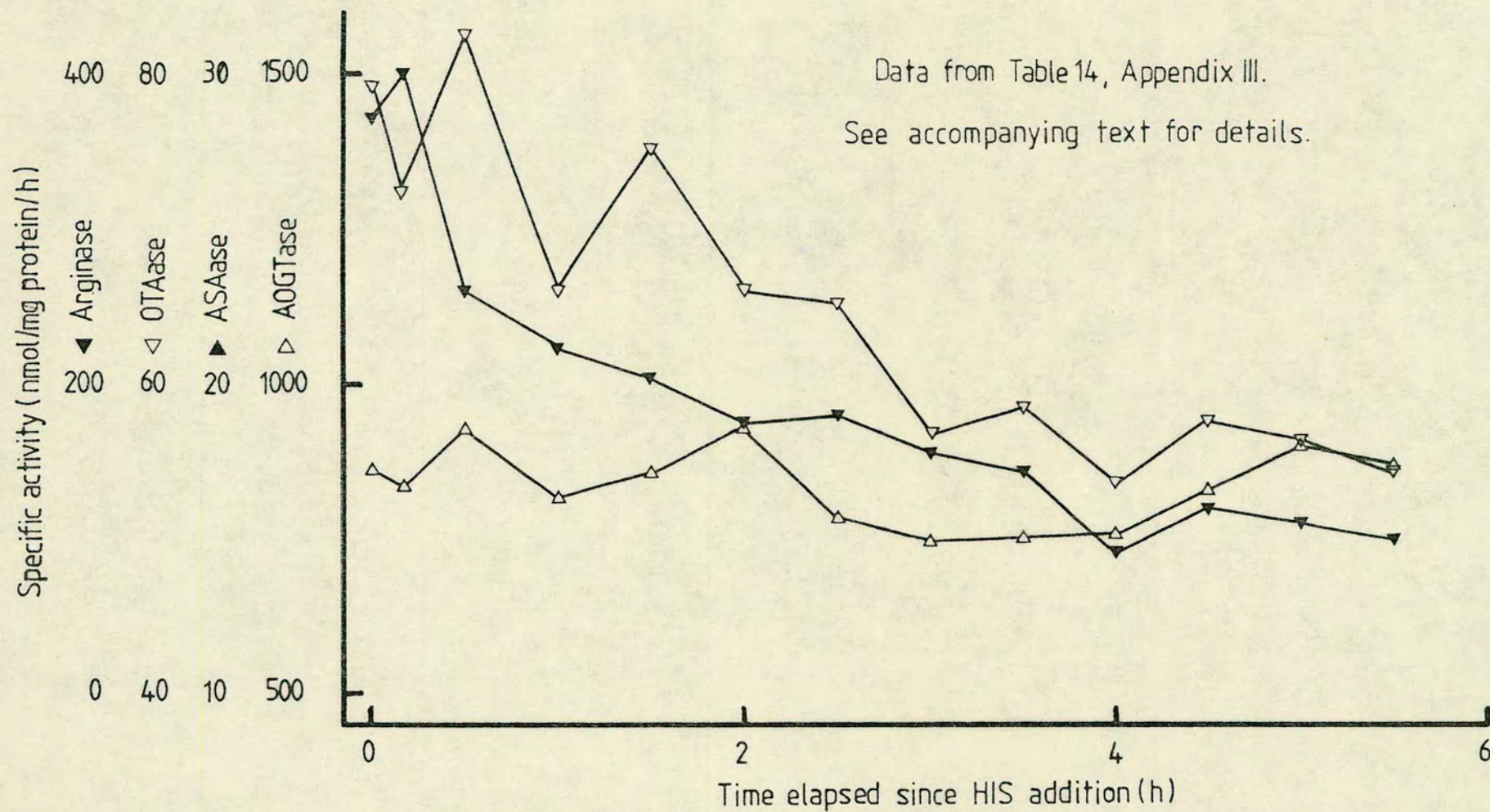
Following the addition of exogenous HIS the mycelial CIT pool falls rapidly. After about $3\frac{1}{2}$ h in a quasi-steady state value of approximately $0.3 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ is approached.

c) Prior to the addition of HIS to the medium, the mycelial ARG pool in CIT-grown cultures is characteristically high. Following the addition of exogenous HIS the mycelial ARG pool falls with time, but at a rate significantly slower than that for the CIT pool. In principal, rates of change of different pools will be different because the flows into and out of each pool are different. The apparent lag of ARG is therefore not indicative that the two pools are not at steady state with respect to each other. We have already seen that under defined steady state conditions the ARG to CIT relation is distinctly non-linear (Figure 22). The steady stateness of the "run down condition" will depend on the relative rates of change in CIT and ARG at their instantaneous values. This will be discussed later in the text.

Although the rate of fall of the ARG pool significantly decreases with time elapsed since HIS addition it is still falling, though at a low rate, and has probably not settled to a final steady state value. At the end of the experimental time course the ARG value is $2.0 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$, about that characteristic of minimally grown wild-type Neurospora.

d) In contrast to the dramatic movements observed in the HIS, CIT and ARG pools the ORN pool remains relatively constant for the first $3\frac{1}{2}$ h following the addition of HIS to the medium. The ORN

Figure 29: Experiment Xa: the post-HIS addition changes in enzyme specific activities.



pool then rises slowly but steadily throughout the remainder of the experimental time course.

Amongst the other pools monitored minor variations are observed over the experimental time course. The GLU pool shows almost a 2-fold variation amongst the fourteen cultures, but no directional changes are discernable, nor can this variation be correlated with variation in any other known system component. GLY and LYS both show a small but probably significant increase over the experimental time course. This must be presumed to be an indirect or consequential effect of HIS supplementation rather than a function of culture age since no such increase was seen in the culture incubated in medium supplemented only with CIT, and harvested at the end of the experimental time course.

Perhaps the most significant pool movement, outwith those of the ARG pathway intermediates themselves, is that of PRO which is observed to closely parallel the change in ORN. This is consistent with the metabolic relation between these two pools (Figure 1) and the positive correlation noted in previous experiments (Tables 7 and 9).

iii) Enzyme specific activities

The specific activity of the two catabolic enzymes, ornithine transaminase (OTAse) and arginase, and the biosynthetic enzyme, acetylornithine glutamate transacetylase (AOGTase) was determined in each of the experimental cultures. The data are plotted against the time elapsed since HIS addition in Figure 29.

The figure shows that the activities of OTAase and arginase fall in parallel throughout the experimental time course, falling rapidly at the beginning and more slowly towards the end.

Although by no means definitive, this response is not inconsistent with a causal relationship existing between the ARG pool and the coordinate activities of these enzymes, as was suggested by the data from Experiment VIIa (Figure 14 and 15).

In sharp contrast, while showing some variation, the specific activity of AOGTase does not show any significant directional change within the experimental time course. This is again consistent with the data from Experiment VIIa (Figure 16) which revealed no change in the activity of AOGTase until the ARG pool fell to below $2.0 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$, the condition shown to be necessary for the derepression of the biosynthetic pathway enzymes (Barthelmess et al., 1974; Cybis and Davis, 1975). It will be recalled that in Experiment VIIa mycelial ARG pools significantly lower than $2.0 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ were observed under a regime of HIS:CIT supplementation identical to that imposed here. These lower pools may be explained by the longer periods of time elapsed since the addition of HIS before harvesting the cultures in Experiment VIIa compared with this experiment. Certainly there is no indication here that the ARG pool has settled to a final steady state value. Since it was of particular interest to analyse the system response to modulation of the ARG pool at values below $2 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ it was decided to repeat the above experiment but extend the post-HIS addition time course to see whether or not ARG pools in the "derepression range" could be attained.

Figure 30: Experiment Xb: growth rate.

Data from Table 15, Appendix III. See accompanying text for details.

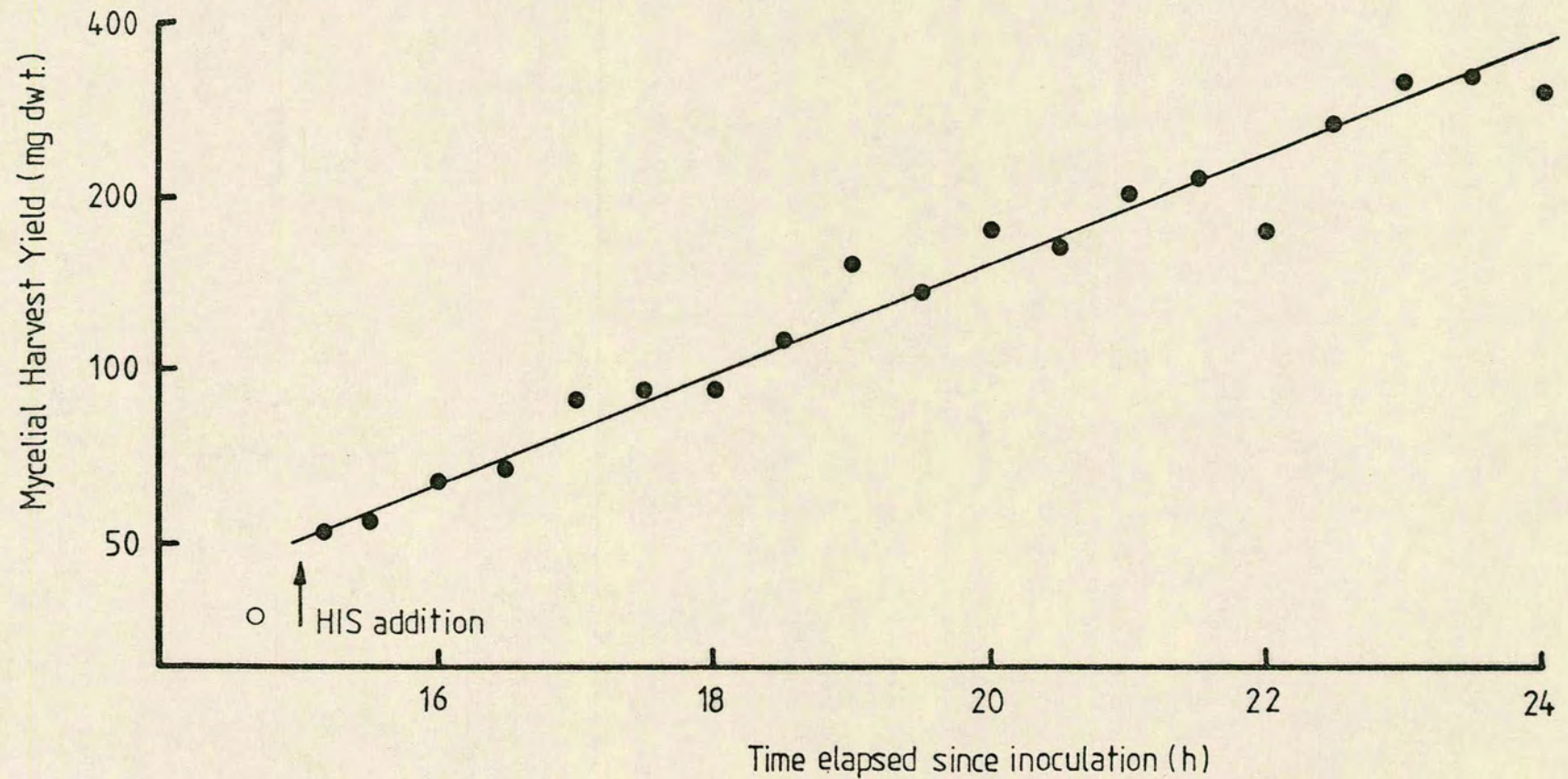
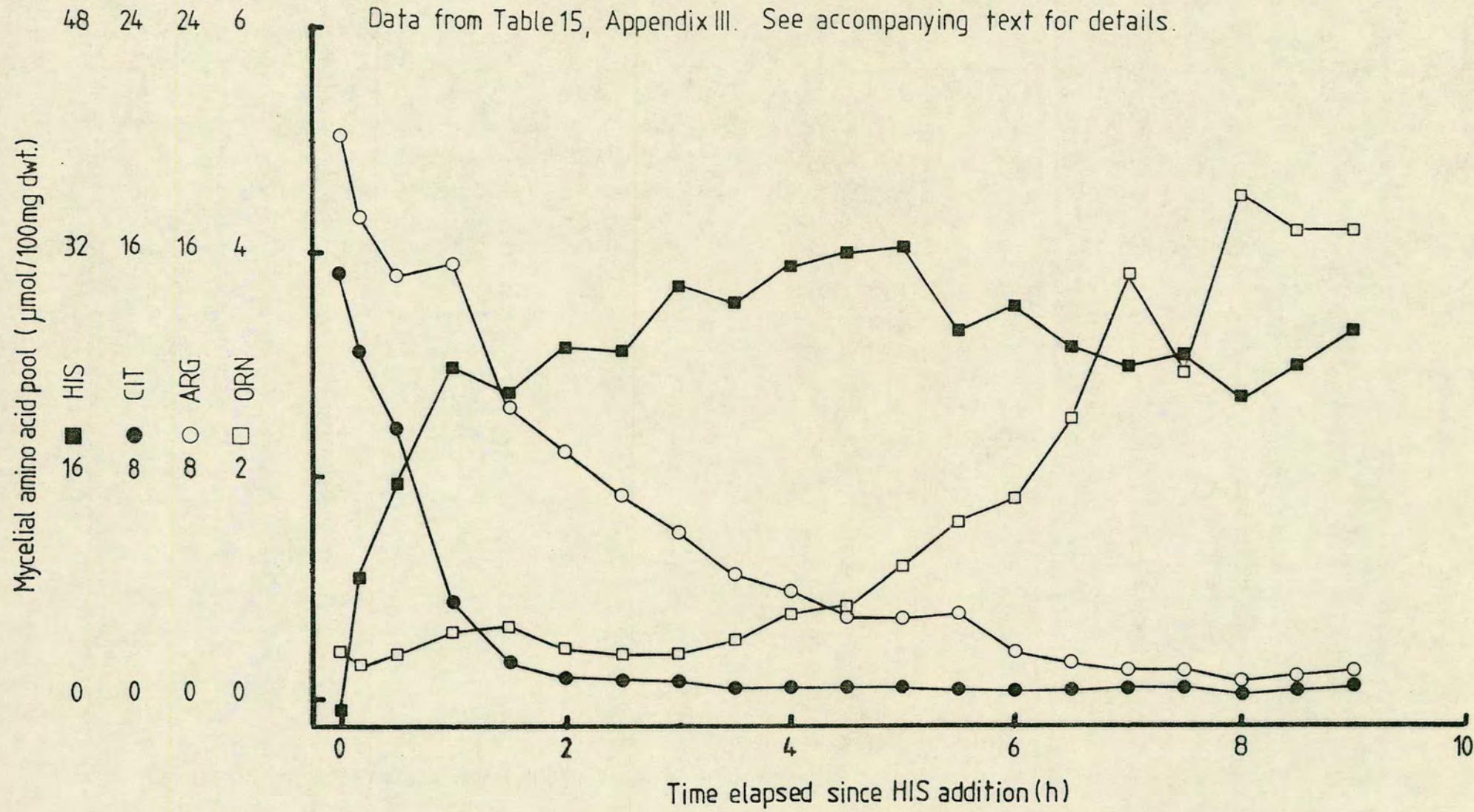


Figure 31: Experiment Xb: the post-HIS addition changes in mycelial amino acid pools.



4.3.2

Experiment Xb: further post-HIS addition changes in mycelial aminoacid pools and enzyme specific activities in *arg-12* ure

The details of this experiment and the results obtained are shown in Table 15. The experiment was an exact repeat of Experiment Xa but with the monitoring of pools and enzymes continued until 9 h after HIS addition. The salient features of the data are as follows:

(i) Growth rate

Figure 30 shows that the cultures grew at a constant exponential rate throughout the entire experimental time course. However, the doubling time of 3 h 5 min is significantly longer than the 2 h 15 min observed in Experiment Xa under the same level of HIS:CIT supplementation. The only difference between the two experiments which accounts for this growth rate effect is the earlier (15 h) post-inoculum addition of HIS in this experiment compared to 17 h in the previous. We have already seen the dramatic effect of HIS on growth when added at, or soon after, inoculation (Tables 1 and 2). A small effect appears still to remain even when addition is delayed by 15 h (see Figure 10).

(ii) Mycelial aminoacid pools

The complete data on mycelial aminoacid pool levels in the cultures are presented in Table 15. The mycelial HIS, CIT, ARG and ORN pools are plotted against the time elapsed since HIS addition to the medium in Figure 31.

Comparison with the corresponding Figure 28 for Experiment Xa shows that the changes in pools observed during the first 5½ h after HIS addition are almost exactly the same in the

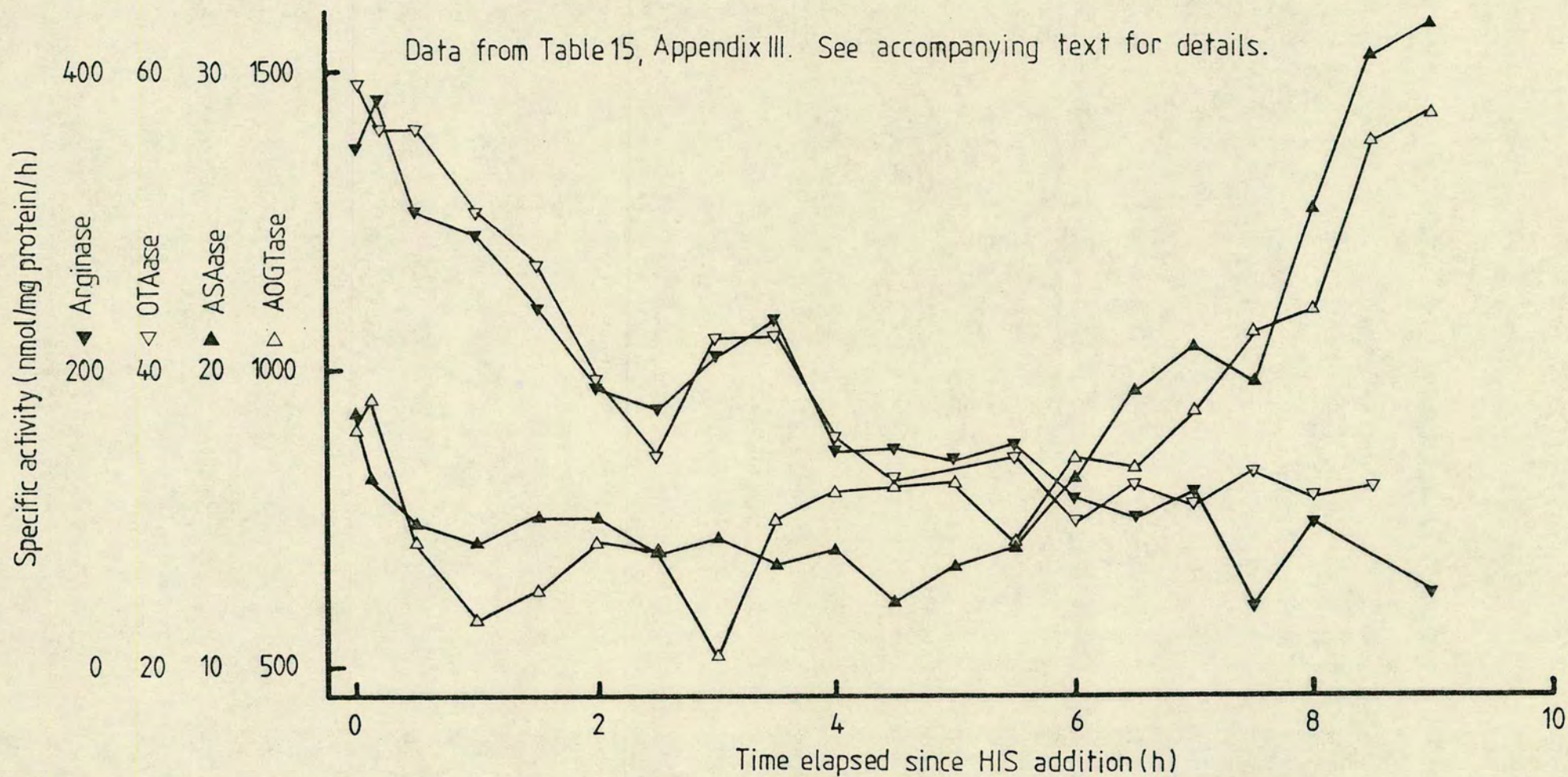
two experiments. Not only does this mean that the conclusions drawn from the observations made in experiment Xa are substantiated but also it provides very good evidence for the reliability and repeatability of the experimental methodology. The findings of Experiment Xa are, however, extended by the observations made here of further pool changes beyond $5\frac{1}{2}$ h post-HIS addition. The pool behaviour may be summarised as follows:

a) As in Experiment Xa, the mycelial HIS pool expands rapidly immediately following the addition of HIS to the medium, reaching a plateau value at about the same time after HIS addition. However, it is interesting to note that the maximally expanded level of HIS observed in this experiment was significantly lower than that observed in Experiment Xa. This behaviour is consistent with the conclusion drawn from the comparative growth rates that an age-dependent HIS effect on growth and, coincidentally, development or expression of HIS transport activity is still felt even if HIS addition is delayed until 15 h after inoculation (see Figure 10).

b) the post-HIS addition change in the CIT pool over the corresponding time course, is almost identical to that observed in Experiment Xa with the CIT continuing to remain at a quasi-steady state value of about $0.3 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ for the rest of the experimental time course.

c) The change in the ARG pool over the first $5\frac{1}{2}$ h following HIS addition is almost identical to that observed in Experiment Xa. However, the ARG pool continues to fall to values below $2.0 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ with increasing time elapsed since HIS addition, as predicted, reaching a quasi-steady state value of about $1.0 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ some 7 h after HIS addition.

Figure 32: Experiment Xb: the post-HIS addition changes in enzyme specific activities.



d) As observed in Experiment Xa, about a three-fold rise in ORN is seen well before the ARG pool enters the derepression range. However, the ORN pool rises dramatically as soon as the ARG pool falls to below $2 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ The ORN pool expansion appears to level off at the same time as the ARG pool settles to its final quasi-steady state.

e) The PRO pool agains shows a strong positive correlation with the ORN pool but the PRO pool response levels off while the ORN pool continues to rise. This may indicate the saturation of the OTAase enzyme at these higher ORN pools, but other explanations could be advanced. The PRO response, as an indicator of the OTAase flux, will be discussed more fully later in the text.

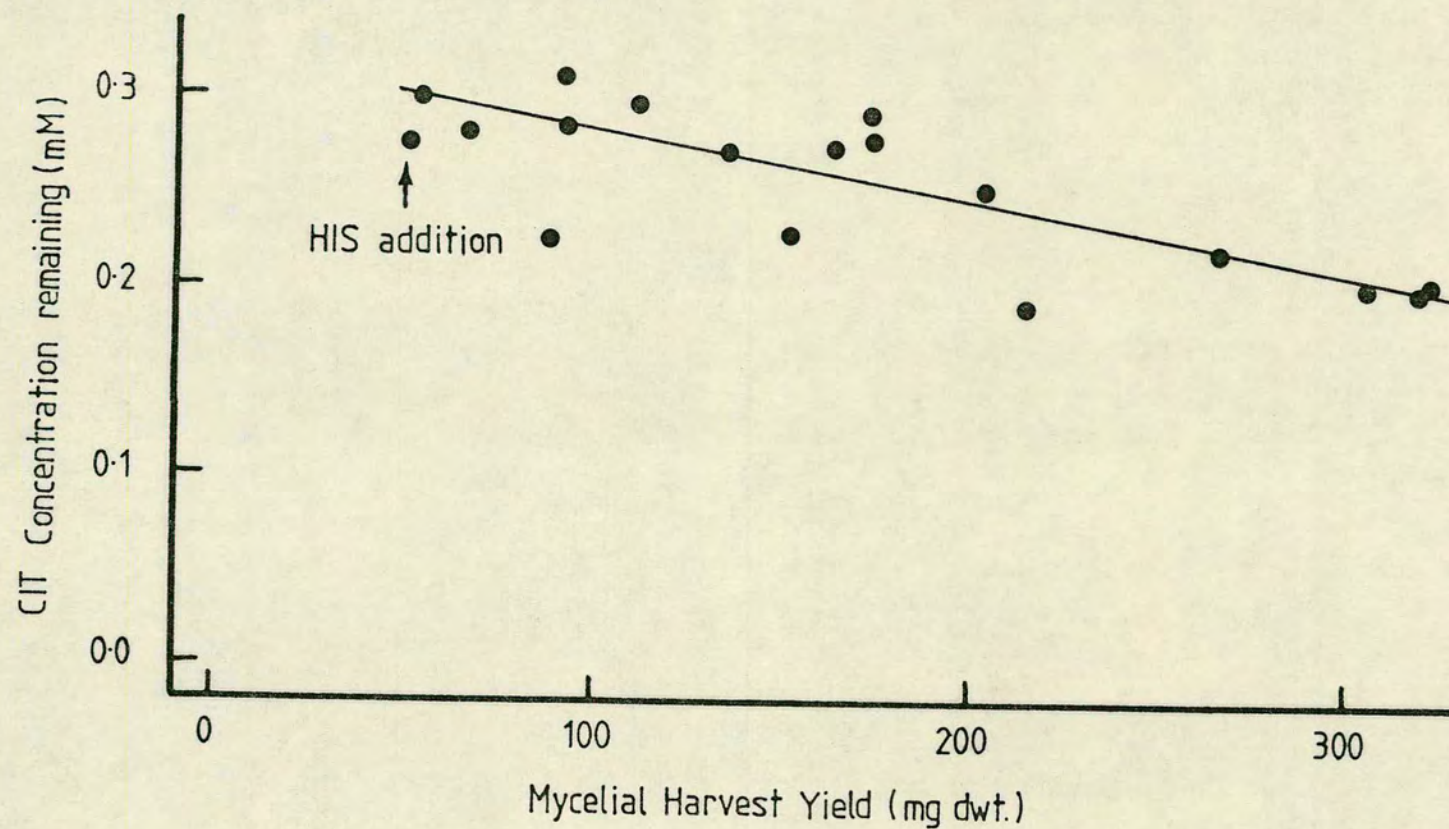
(iii) Enzyme specific activities

The activity of a second biosynthetic enzyme, arginino succinase (ASAase), was assayed in this experiment in addition to the three enzyme activities analysed in Experiment Xa. Their specific activities are plotted against the time elapsed since HIS addition in Figure 32. The coordinate fall observed in Experiment Xa in the specific activities of arginase and OTAase is repeated here and continued throughout the extended experimental time course.

The activities of the two biosynthetic enzymes, AOGTase and ASAase, are also coordinately modulated but, unlike the catabolic enzymes, no significant directional change in specific activity is observed in the first $5\frac{1}{2}$ h following the addition of HIS to the medium. This is in agreement with the analyses of AOGTase specific activity in cultures from Experiment Xa. The three-fold rise in ORN observed over this period is therefore due to a deinhibition at constant enzyme quantities. From 6 h after HIS addition onwards,

Figure 33: Experiment Xb: the rate of medium CIT depletion.

Data from Table 15, Appendix III. See accompanying text for details.



however, both specific activities increase dramatically and in parallel, possibly beginning to level off at the last time point. Over this 3 h period both specific activities doubled. As predicted, the initiation of the increase in these enzyme activities correlated closely with the fall in the ARG pool to values below $2.0 \mu\text{mol} \cdot 100 \text{ mg}^{-1}$ d.wt. The further increase observed in the ORN pool, indicative of an increase in flux through the early steps of the ARG pathway, is consistent with this increase in AOGTase specific activity determined in vitro. However, the two-fold derepression of the AOGTase specific activity can account for, at most, a two-fold increase in the flux to ORN; over the corresponding time the ORN pool actually increases about three-fold. Thus, unless the novel assumption is made that derepression is not co-ordinate but that another, "rate limiting" enzyme involved in ORN synthesis is derepressed by more than three-fold, it can be concluded that the additional fold increase in ORN must reflect the additional effect of another regulatory phenomenon, presumably further deinhibition by ARG of ORN synthesising activity.

(iv) Medium CIT depletion

Medium samples were taken immediately before each growth flask was harvested and the concentration of CIT remaining determined using BUN colorimetric reagents by the method described in Section 2.2.8.1. The results of this analysis are shown in Table 15 and plotted against the mycelial harvest yields in Figure 33. The figure shows that a rate of CIT depletion of about $15 \mu\text{mols. per } 100 \text{ mg dry weight increase}$ was established immediately following the addition of HIS to the medium, and which continued for the rest of the experimental time course. It will be shown in Section 4.3.7.1 that the essentially

invariant pool regime reached at 7 h after HIS addition (i.e. cultures CH 15 to CH 19) must represent the final steady state in response to this experimentally controlled rate of CIT uptake.

In this and the preceding experiment the reliability and repeatability of the experimental methodology and the analytical techniques have been established. However, it remains to be established whether or not the system is at a quasi-steady state throughout the post-HIS addition pool adjustments, albeit a continually changing steady state. This question and the conclusions with regard to the control of flux which may be drawn from the pool and enzyme correlations described by the data from both experiments will be discussed in the next section.

4.3.3 Experiments Xa and b: the quasi steady state of the "run down" condition and the control of flux.

4.3.3.1 Correlations between mycelial aminoacid pools

In Section 4.2.6 two important relations between ARG pathway intermediates observed under steady state conditions were discussed, namely the positive, non-linear relation between CIT and ARG (figure 22) and the negative non-linear relation between ARG and ORN (Figure 24). If these same two correlations, as described by the data from Experiments Xa and b, agree closely with those observed under defined steady state conditions then it may be confidently concluded that in such "run down" experiments the system is at an instantaneous, quasi-steady state at each point in time following the addition of HIS.

Figure 34: The “run down” relation between CIT and ARG in arg-12 ure.

Data from Experiments Xa(○) and b(●), Tables 14 and 15, Appendix III. See accompanying text for details.

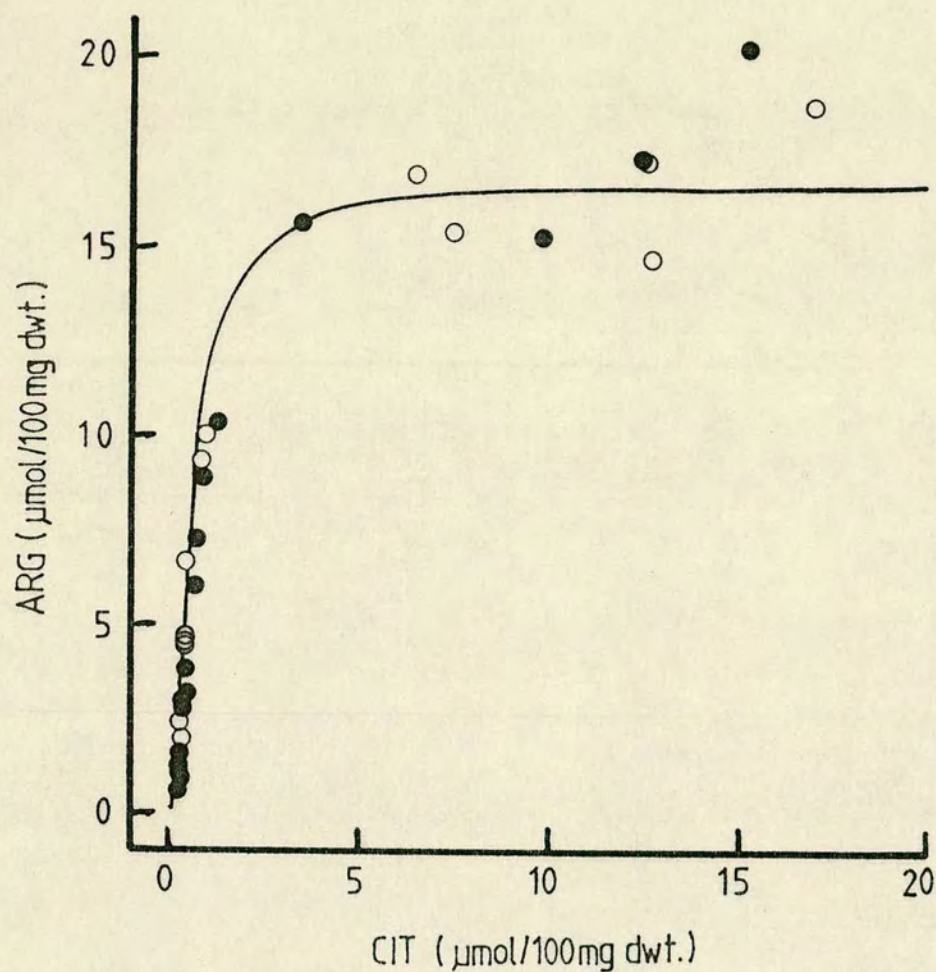


Figure 35: The linear part of the "run down" relation between CIT and ARG in arg-12 ure.

Data from Experiments Xa (○) and b (●), Tables 14 and 15, Appendix III. See accompanying text for details.

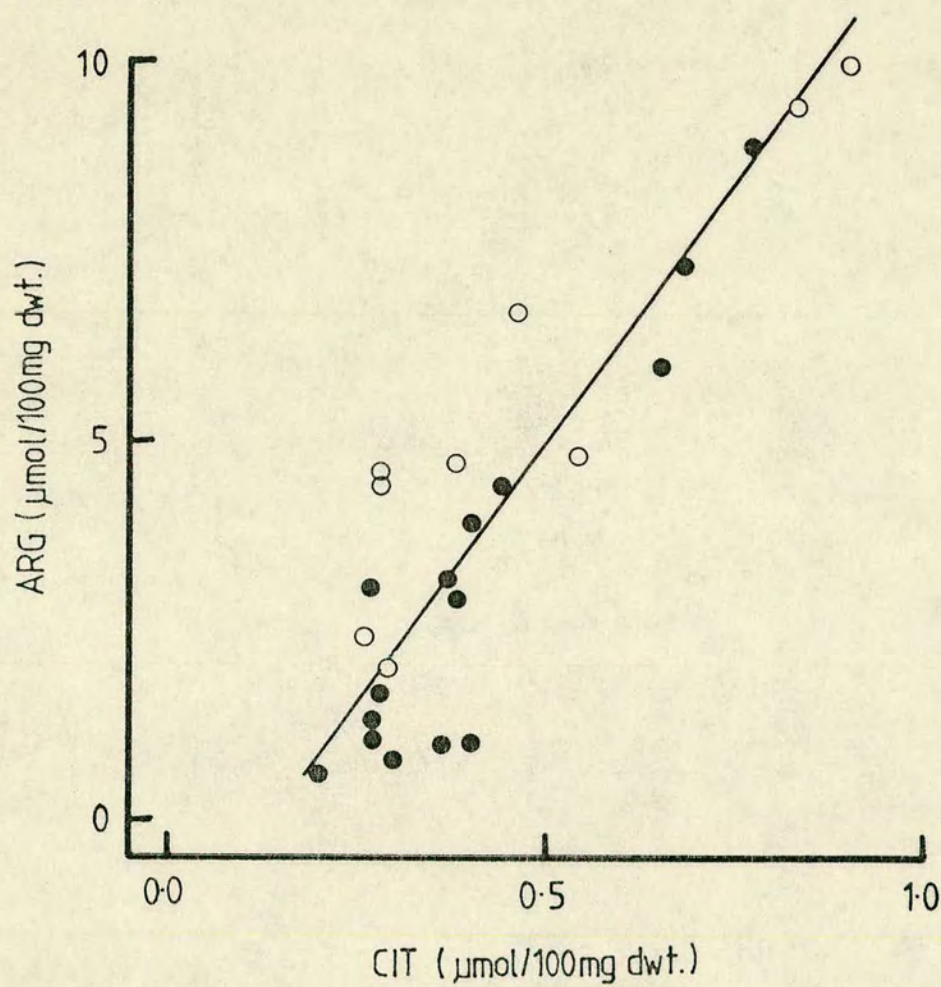
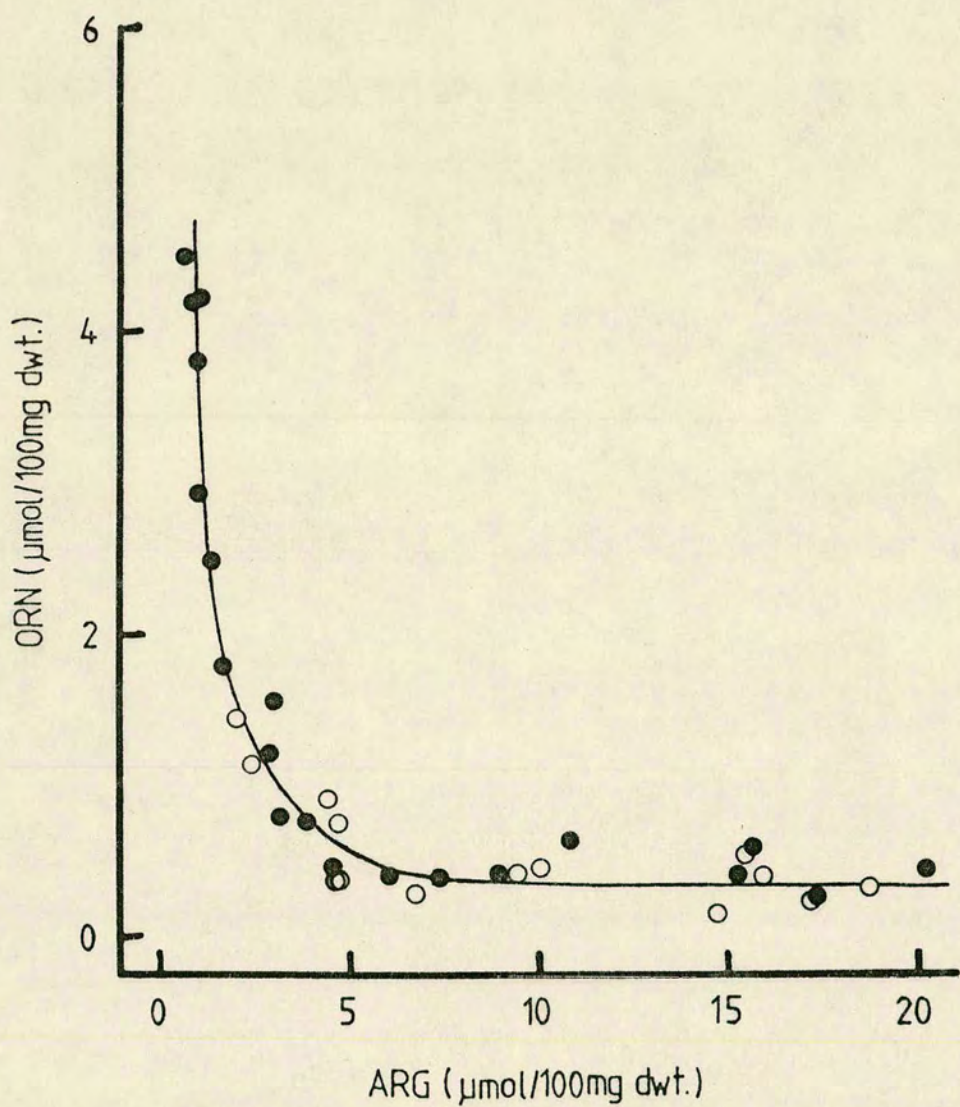


Figure 36: The "run down" relation between ARG and ORN in arg-12 ure.

Data from Experiments Xa (○) and b (●), Tables 14 and 15, Appendix III. See accompanying text for details.



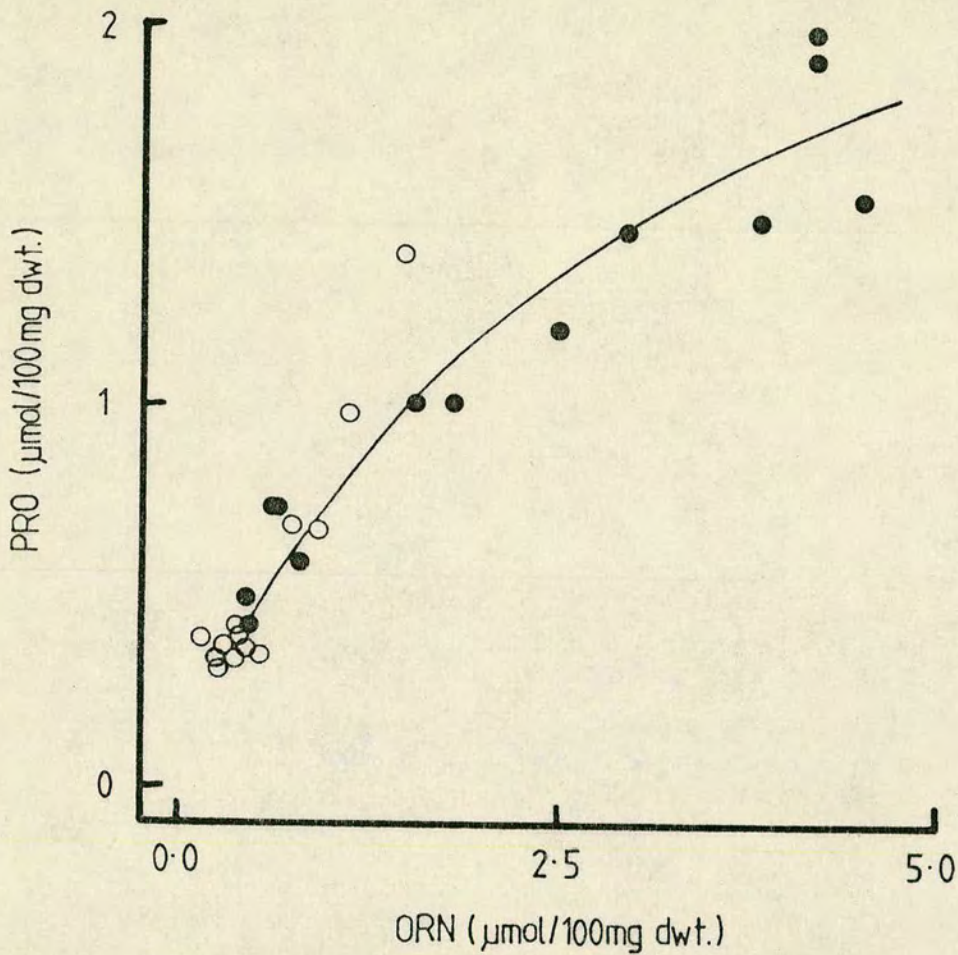
In Figure 34 the ARG pools observed in Experiments Xa and b are plotted against the corresponding CIT pool; the dashed line is taken from Figure 22, where the corresponding data from the "steady state" experiments were plotted. Thus, Figure 34 shows that the relation between the instantaneous CIT and ARG pools described in the "run down" experiments agrees very closely with the same pool relation defined by established steady states. An expansion of the linear part of the relation is shown in Figure 35.

Similarly, Figure 36 shows a close agreement between the ORN versus ARG pool relation described by the "run down" experiments, Xa and b, and the same pool relation as defined by the data from the steady state experiments, VIa, VIIa and VIII (Figure 24).

From the close agreement observed between the "run down" and the "steady state" relation for both pool correlations we may conclude that in such "run down" experiments the system as a whole adjusts closely to an instantaneous steady state throughout the post-HIS addition pool and flux changes. This must mean that the rate of change in the CIT pool (following the sudden reduction in the rate of uptake consequent upon HIS-addition) is significantly slower than the relaxation times of the subsequent metabolic transformations. It also implies that the relaxation times of the fluxes into and out of ORN are significantly higher than the rate of change in the ARG pool and that the response of ORN to the effector role of decreasing ARG is essentially immediate. The question of the relative contributions to the ORN pool of feedback inhibition and derepression of the proximal enzymes by ARG has already been introduced. This question will be returned to after the correlations between the pathway enzymes and pools have been described and discussed.

Figure 37: The "run down" relation between ORN and PRO in arg-12 ure.

Data from Experiments Xa(○) and b(●), Tables 14 and 15, Appendix III. See accompanying text for details.



Several types of "interactions" can be invoked to explain the form of the ARG to CIT pool relation. For instance, the ARG pool could be effectively "locked" at a maximal level by the feedback inhibition and/or repression by ARG on the activity of one or both of the enzymes involved in the transformation of CIT to ARG (Kacser and Burns, 1973). Alternatively, the relationship may reflect the induction and/or activation by ARG of ARG-utilising enzymes. Finally, the relation may arise quite independently of changes in enzyme activity but simply through the saturation of SYNase and/or ASAase at higher levels of mycelial CIT. We shall return to the question as to which explanation is most favoured after the correlations between relevant pools and enzyme specific activity have been described and discussed.

A final correlation of interest to the control of flux is that between the ORN and PRO pools shown in Figure 37. The positive relation between the two suggests that under these conditions the flux through OTAase is controlled principally by the mass action effect of ORN. This question will be returned to after the modulation of OTAase activity has been analysed further.

4.3.3.2

Correlations between mycelial aminoacid pools and enzyme specific activities

a) The control of the specific activity of the biosynthetic enzymes, AOGTase and ASAase

We have already noted that the specific activity of AOGTase remains substantially constant throughout the time course of Experiment Xa whereas this activity and also that of ASAase showed

Figure 38: The "run down" relation between ARG and the specific activity of AOGTase and ASAase in arg-12 ure.

Data from Table 15, Appendix III.

See accompanying text for details.

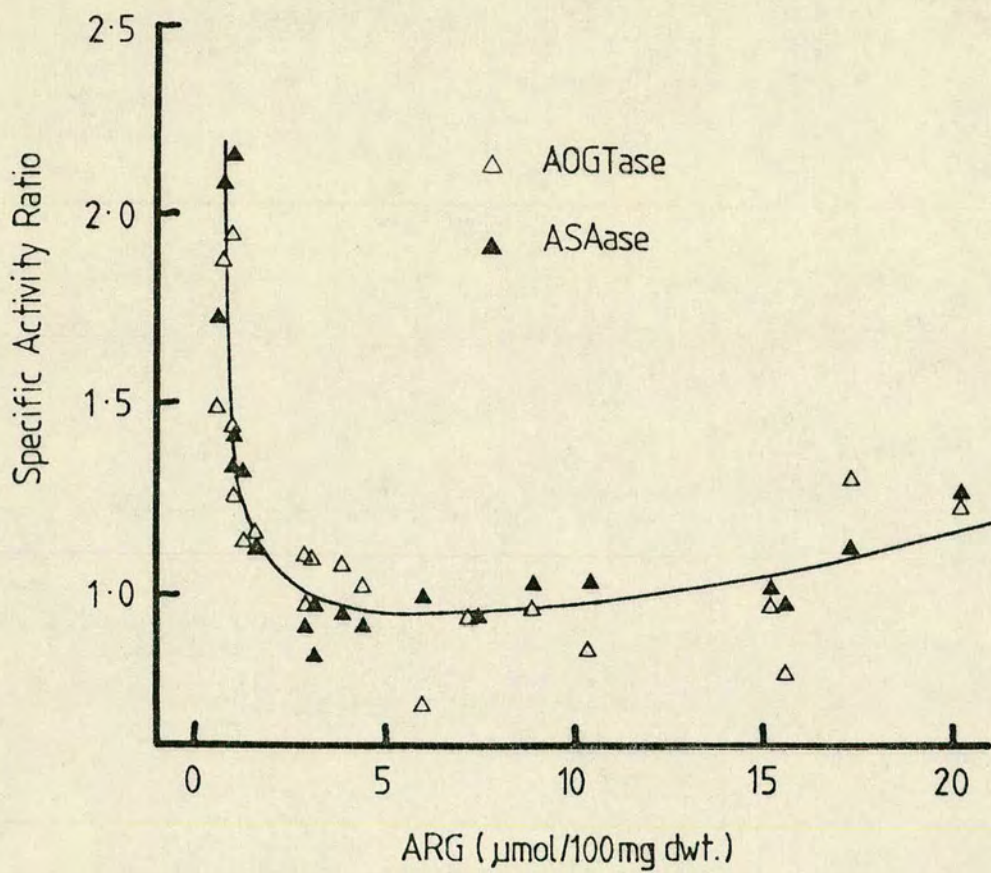
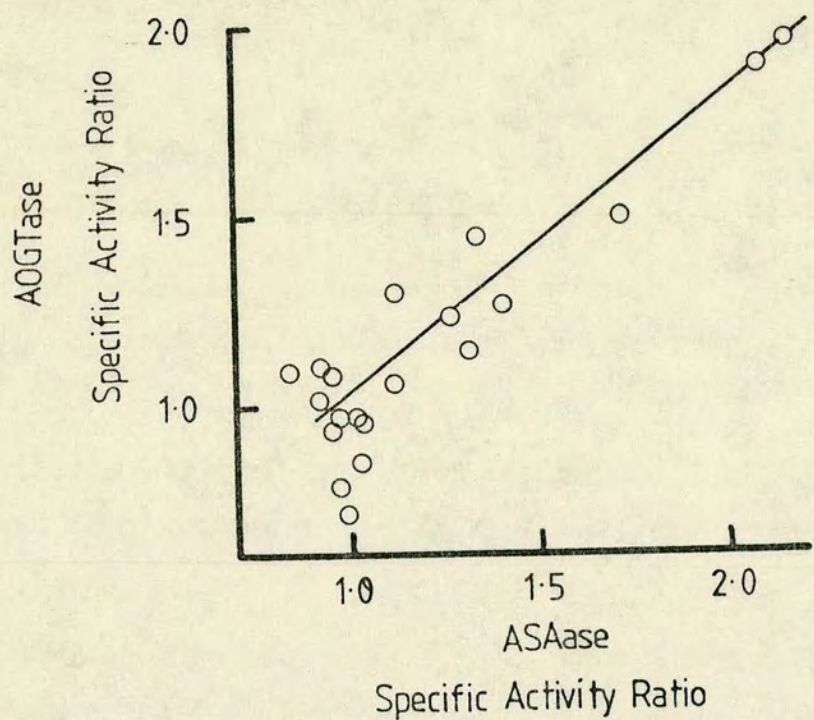


Figure 39: The relation between the specific activity ratios of ASAase and AOGTase.

Data from Table 15, Appendix III.

See accompanying text for details.



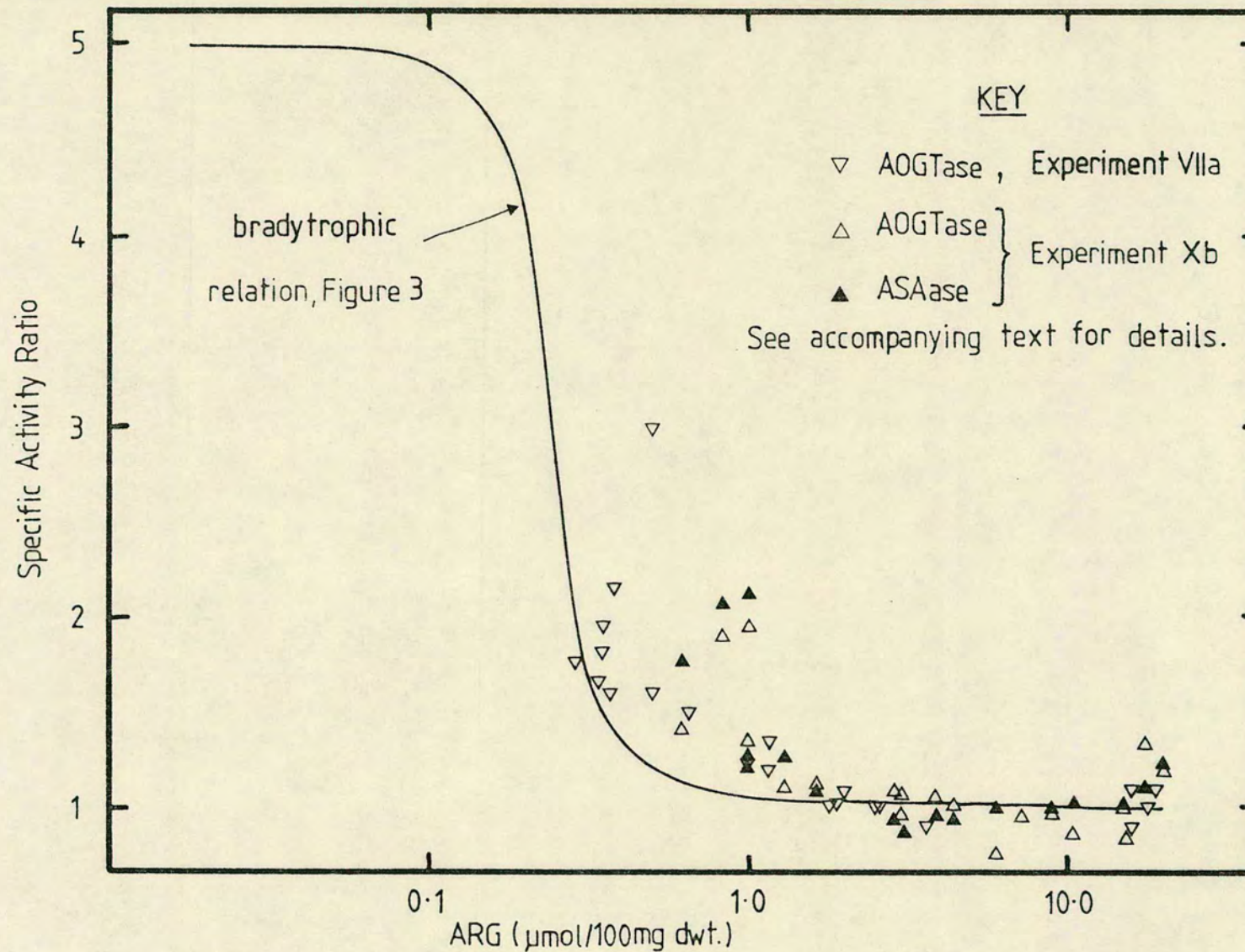
a sharp 2-fold increase towards the end of the time course in Experiment Xb (Figures 29 and 32 respectively). The enzyme data from this latter experiment (expressed as the ratio of each specific activity over the mean specific activity at ARG pools greater than $2 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$) are plotted against the corresponding ARG pools in Figure 38. The data describe a derepression curve, with both specific activities increasing sharply as the ARG pool falls to values below $2.0 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$, essentially the same as that defined by the corresponding data from the steady state experiment VIIb (Figure 16). This observation implies that the derepression system responds with a new rate of enzyme synthesis at much the same rate as the ARG pool falls.

The coordination of derepression is shown by the linear plot of AOGTase SAR versus ASAase SAR with a slope of about unity (Figure 39).

In neither the "steady state" experiment, VIIb, nor these two "run down" experiments is there evidence for a maximal level of derepression having been reached. This is perhaps not surprising since Barthelmess et al (1974) did not observe the derepression curve to plateau until the ARG pool had fallen to below a tenth of the wild-type minimal value and the specific activity increased five-fold. It is noteworthy that in the present experiments the ARG pool did not fall substantially below $1 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ (half the wild type MIN value) except in certain cultures where growth had clearly departed from exponentiality. The very small pools which can be observed in certain minimally-grown

bradytrophic strains (Barthelmess et al, 1974) were never obtained. This may be explained by the fact that as the ARG pool falls and the biosynthetic enzymes are derepressed so the flux to ARG will tend to increase. The ARG pool will, therefore, be buffered against further change, by virtue of the allosteric rates of the derepression, with the ARG pool held at around the maximum slope on the curve. As can be seen in the present case, where the rate of CIT input is controlled by HIS inhibition and both ASAase and SYNase have wild-type kinetic parameters, this buffering effect is considerable. In the bradytrophic strains of Barthelmess et al (1974) the flux to ARG is effectively limited by the low activity of the revertant enzyme. Despite derepression the specific activity of the revertant enzyme is very low. Consequently, the Sensitivity Coefficient of the enzyme is nearly one and, therefore, the enzyme may be said to be "controlling". The inhibition of CIT uptake in arg-12 is analogous to modulation of OTCase specific activity; we would observe the same type of systemic response as described here consequent upon HIS addition if it were possible to replace the wildtype OTCase with a partially revertant enzyme and monitor the time course of subsequent pool changes in minimal medium. However, we have not even succeeded in achieving the reduction in flux to ARG imposed by arg-12^S (where the specific activity of OTCase is 3%, the ARG pool is 10% and the derepression about 400% of the minimally grown wild-type values (Barthelmess et al, 1974)) by inhibition of CIT uptake under exponential growth conditions in arg-12. This is because as the ARG pools fall into the "derepression range" the difference between the balanced growth

Figure 40: The effect of HIS on derepression in arg-12 ure.



and a growth rate limiting flux to ARG becomes progressively less; the flux to expansion of the ARG pool itself becomes a progressively smaller absolute amount and proportion of the total flux to ARG whereas the flux to protein necessary for balanced growth, while remaining a constant, becomes an increasingly large proportion of the total flux. The small degree of derepression but high degree of buffering capacity observed here are both the direct consequence of the relatively small reduction (compared with the wild-type minimal) in the flux to ARG mediated by uptake inhibition. The Sensitivity Coefficients of all the steps including the "controlled" step, are therefore almost equally low and no one step can be flux "limiting" or "controlling". A further reduction in the quasi-steady state ARG pool to below the values seen at the end of the "rundown" experiment Xb (Figure 32) will require a very small increase in the degree of CIT uptake inhibition imposed; any more and the flux to protein would become growth rate limiting. Such a departure from exponentiality is seen in the tail end of the "steady state" Experiment VIIa (Figure 13) at HIS:CIT ratios greater than 5:1. Certainly, the same "fine" control imposed by revertant enzymes on the flux to ARG is potentially within the capacity of CIT uptake inhibition approach but would require repeated and marginal variation of the medium HIS:CIT ratio.

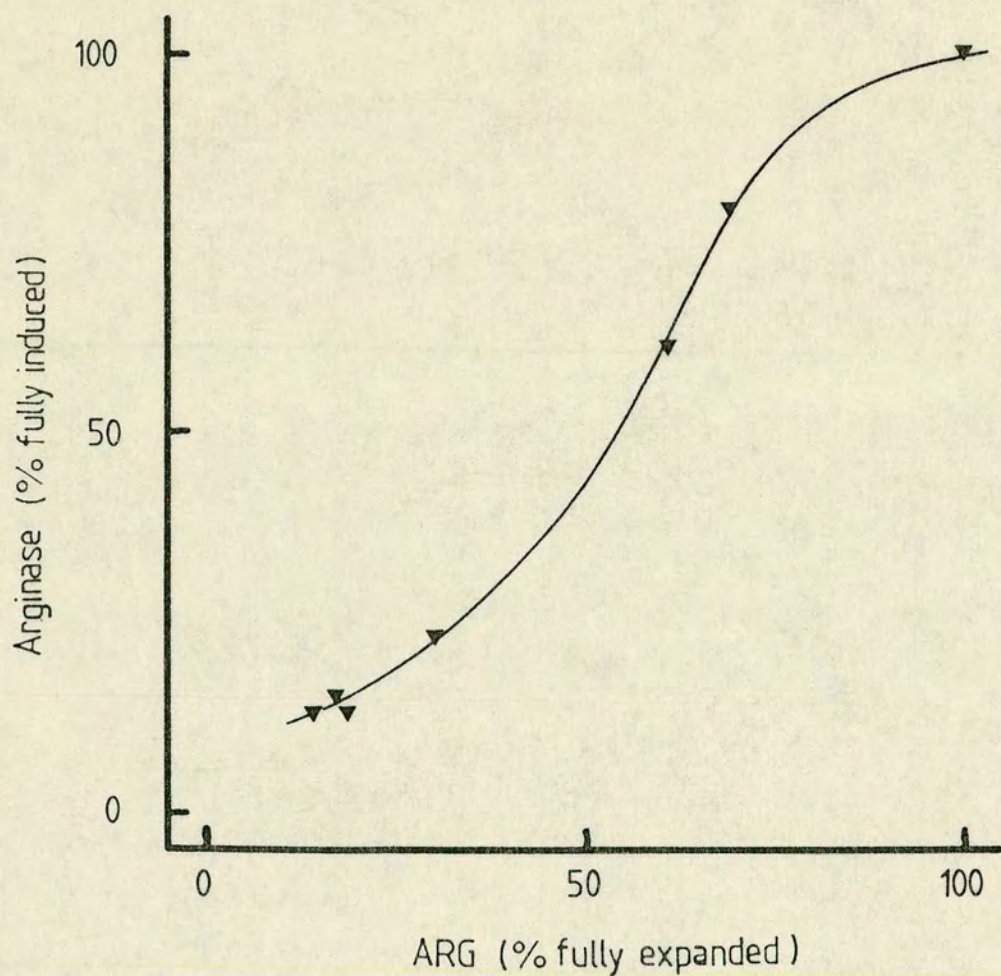
An interesting phenomenon is revealed when the enzyme data from the "steady state" Experiment VIIa (Table 9), and the "run down" Experiment Xb (Table 15) are compared with derepression curve in bradytrophs as shown in Figure 40. The plot shows that when the ARG pool is set by HIS-inhibition of CIT uptake, as opposed to

genetic modulation of the biosynthetic enzyme activities, the derepression curve is shifted to the right, i.e. a greater degree of derepression is observed for an identical total extractable ARG pool. This effect is slight under "steady state" HIS:CIT conditions, but quite pronounced in the "run down" experiment. All the evidence would suggest that the derepression system we are observing under the HIS:CIT condition is the same as in bradytrophi. The shift phenomenon must mean, as has previously been suggested (Barthelmess et al., 1974), that the derepression signal is not the total extractable ARG pool but a ~~sub~~pool or direct derivative thereof. Furthermore, an indirect effect of HIS is to alter the normal distribution of ARG between these subpools of the total extractable.

In fact, this shift is what would be expected on the basis of the indirect effect of HIS on ARG distribution postulated in Section 4.1.6, assuming (as is most probable) that the signal is the cytosolic ARG pool (or a direct derivative thereof). In both the "steady state" and the "run ^{down}" experiments, where we start with a fully expanded vesicular pool, the addition of HIS will cause the total ARG pool to drop, through growth dilution and metabolism, consequent upon the inhibition of exogenous CIT uptake. However, according to the working hypothesis constructed in Section 4.1.6, the rate of ARG egress from the vesicle to the cytosol will also be inhibited to a degree by the accumulating mycelial HIS. Thus, for a given total ARG pool the cytosolic ARG pool and, therefore, the derepression signal will be lower in the presence than in the absence of HIS. There is direct evidence for ORN, another metabolite held in the vesicle, competing with ARG for

Figure 41: The steady state relation between ARG and arginase in ure.

Data from Experiments VIIb and IX, Tables 11 and 13, Appendix III.
See accompanying text for details.



translocation across the vesicular membrane (Davis et al, 1978b). Presumably there will also be a feedback of the ARG-mediated accumulation of ORN on the rate of equilibration of ARG between the vesicle and the cytosol.

There does not appear to be a substantial lag between the attainment of a given ARG pool and the response by the derepression system. If there were, this would tend to shift the "run down" curve to the left of the "steady state" curve. The more pronounced shift to the right under the "run down" condition must reflect a lag in the rate of equilibration of ARG between the vesicle and cytosol. We believe that HIS and ORN effect this rate, but the difference between the "steady state" and "run down" derepression curve may be largely due to the normal rate of equilibration between the vesicle and cytosol being lower than the rate of fall in flux to (cytosolic) ARG from CIT. This possibility receives support from observations, Dr. H. Flint, unpublished data, of a similar shift in the derepression curve under ARG-starvation conditions in the absence of HIS or large ORN pools.

b) The control of the specific activity of the catabolic enzymes

(i) Arginase

The arginase activity determined in ure under several different steady state conditions is plotted against the corresponding ARG pool in Figure 41. Clearly there is a very strong positive correlation between the ARG pool level and the level of arginase induction, an observation which implies that ARG, or a direct derivative thereof, is the coinducer for arginase induction

(Castaneda et al., 1967; Weiss and Davis, 1977). Although the paucity of data precludes a rigorous quantitative interpretation, the form of the relation, as drawn, between the ARG pool, i.e. "signal", and the level of induction does appear to be allosteric. This is consistent with the general acceptance of the notion that the binding and dissociation of ligand in repression-induction systems is an allosteric transformation (Burns and Kacser, 1977).

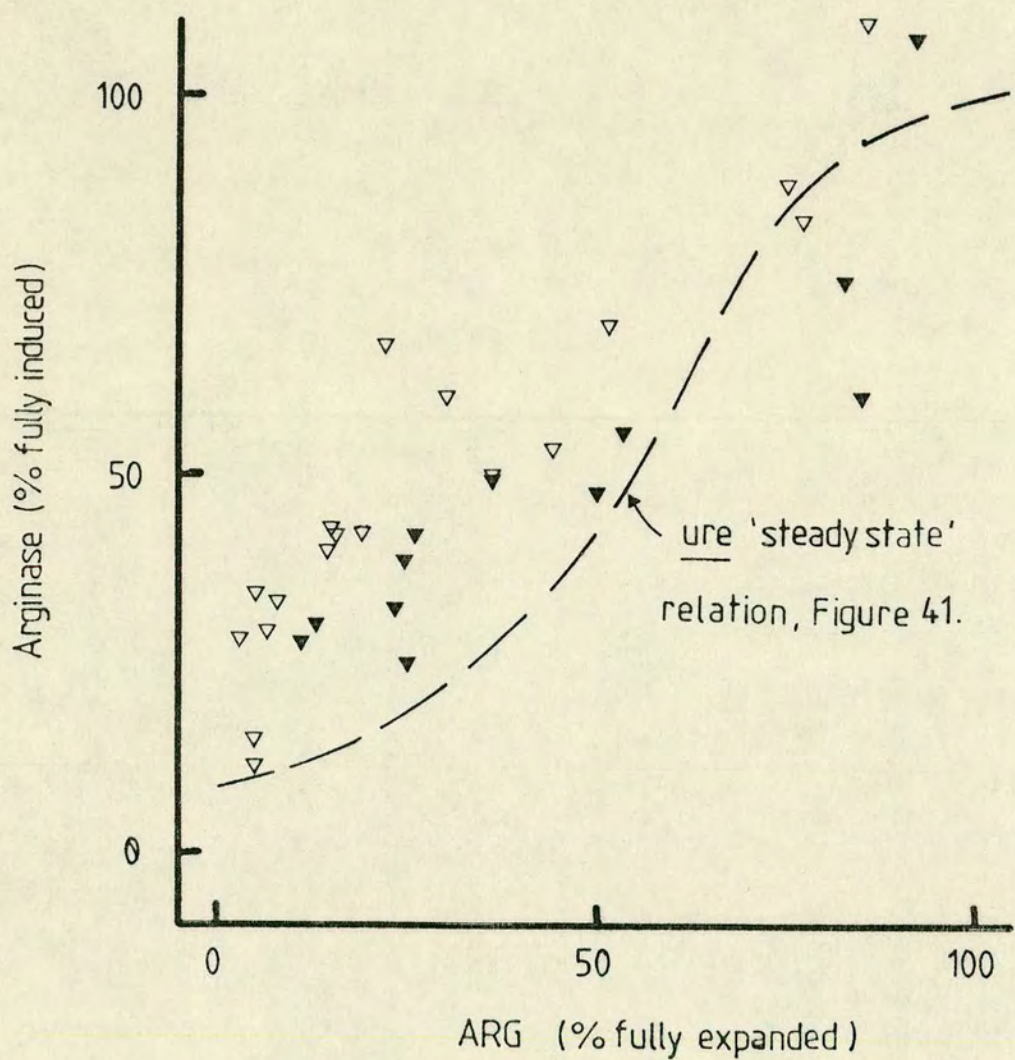
The manner in which the data plotted in Figure 41 were generated merits comment. A maximum level of induced activity was assumed to be present under conditions giving rise to the largest ARG pool, that is on 0.5 mM CIT supplement. The activity and pool levels under the other supplementation conditions are expressed as a percentage of these maxima. The mean specific activity and pool for each steady state condition has been plotted.

It should be noted that differences in absolute, calculated specific activities were sometimes observed (here and elsewhere) between different sets of enzyme assays. These differences are thought to be purely experimental; the relative specific activities for samples grown under different steady state conditions were found to be repeatable when codetermined at different times, with different batches of reagents. Therefore, if absolute specific activities differed between two experiments, as they do here, the conversion of the data from both to comparable units, i.e. percentage of the "fully induced activity", can be justified since the two experiments share a common steady state condition for comparison in the minimally-grown cultures.

A comparable plot of the data from the "run down" experiments (Xa and b) for arginase specific activity (as the percentage of the CIT-only, i.e. "fully induced" level) against the ARG pool (as a

Figure 42: The "run down" relation between ARG and arginase in arg-12 ure.

Data from Experiments Xa (∇) and b (\blacktriangledown), Tables 14 and 15, Appendix III. See accompanying text for details.



percentage of the CIT-only, i.e. "fully expanded", pool) is shown in Figure 42, with the relationship obtained under steady state conditions (Figure 41) superimposed. This figure substantiates the strong correlation between the ARG pool and the level of arginase induction seen under "steady state" conditions (Figure 41).

However, there does appear to be a significant difference between the relationship observed under the "run down" as opposed to the "steady state" condition; for the same level of total ARG the level of arginase induction appears to be generally somewhat higher under the former compared with the latter condition. A hysteresis is indeed expected since under the "run down condition" preformed enzyme must decay before the specific activity determined in vitro reflects the lowered rate of synthesis in vivo in response to a fall in the signal, i.e. ARG pool. The hysteresis observed is however less than expected if arginase specific activity decays simply through growth dilution of enzyme. The more than exponential decay (with respect to ARG pool decay) argues that arginase enzyme is inherently unstable or actively degraded in vivo. (The enzyme is however quite stable in freeze dried mycelial powder where neither degradative mechanism can operate). It is also noticeable that the arginase specific activity starts to fall almost as soon as the ARG pool falls which argues that the time taken to translate a fall in signal level into a new and lower rate of enzyme synthesis is fairly short.

This hysteresis in arginase decay, albeit slight, must have an effect on the arginase flux at a given total extractable ARG pool. That the "steady state" and "run down" ARG to CIT relations are indistinguishable (Figure 34) shows that the

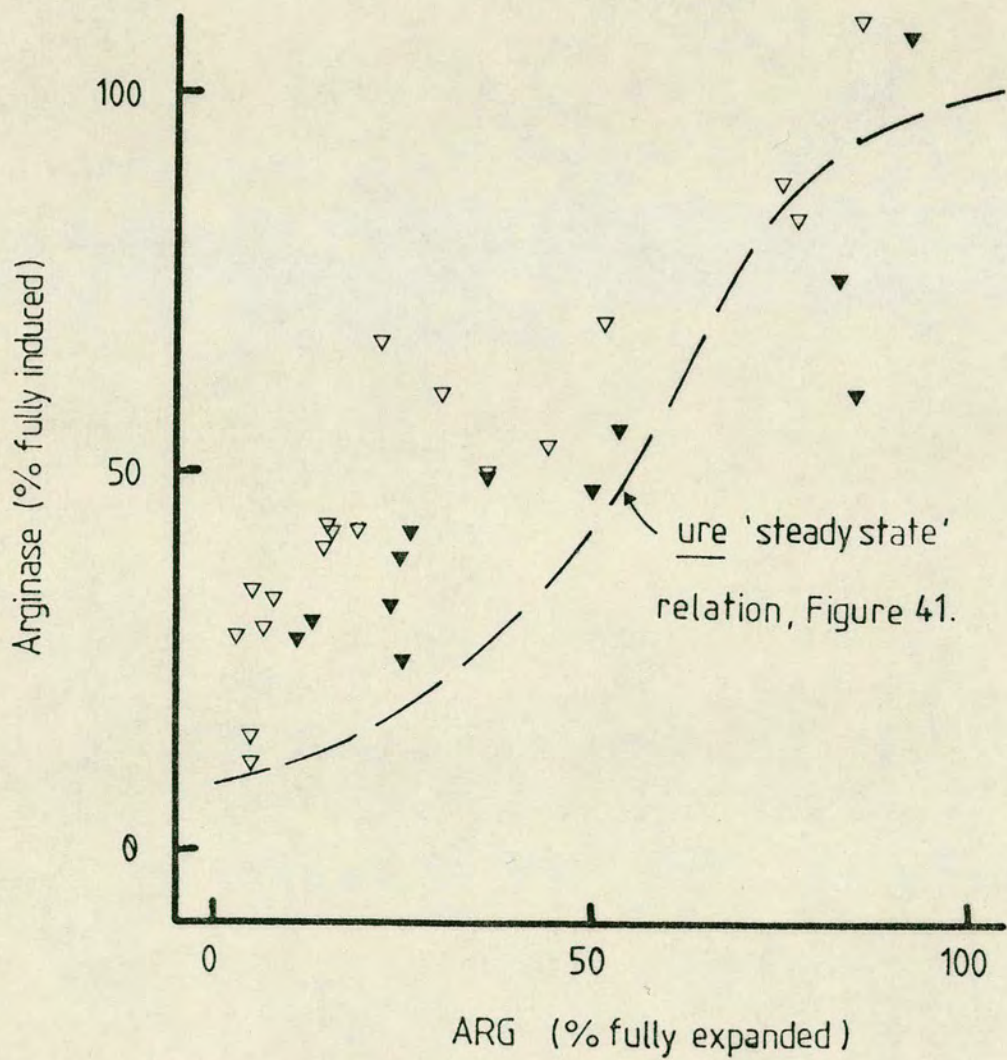
difference in arginase activity has a very small effect on the flux out of ARG.

It will be shown later in the text that no arginase flux is detectable at levels of ARG where derepression is observed. The hysteresis in arginase decay is therefore not thought to contribute to the shift in the "run down" derepression curve to higher total extractable ARG pools compared with the "steady state" condition. Most importantly, at each experimentally determined

How then do the observations and interpretations made here with regard to the modulation of arginase activity compare with those of other investigators? The induction by ARG of arginase is a long established phenomenon (Castaneda et al, 1967). The "role" of arginase induction in the "control" of ARG utilization in Neurospora was the subject of a recent investigation by Weiss and Davis (1977). Following the addition of ARG to the medium of minimally-grown ure cultures, they observed the mycelial ARG pool to increase rapidly, reaching a plateau after about 3 h. In contrast, no increase in arginase specific activity was observed for some 40 to 60 min after the addition of ARG to the medium, after which the activity increased linearly over the remaining time. The authors concluded that arginase induction, which requires protein synthesis, takes place at a constant rate despite a continually expanding ARG pool. Although they say that the specific activity of arginase declines after the depletion of ARG from the medium they do not describe the form of the decay. Their conclusion that induced arginase is synthesized at a constant rate, i.e. by an ARG-mediated "switch" mechanism, is at variance with the

Figure 42: The "run down" relation between ARG and arginase in arg-12 ure.

Data from Experiments Xa (∇) and b (\blacktriangledown), Tables 14 and 15, Appendix III. See accompanying text for details.



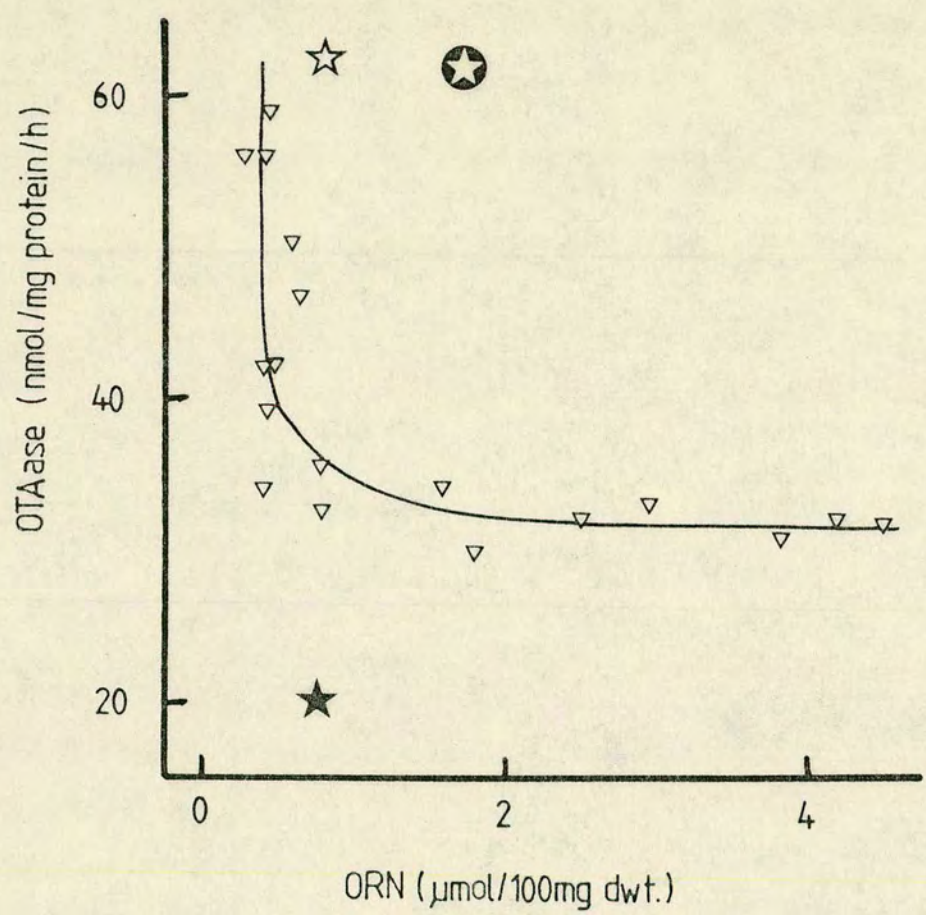
percentage of the CIT-only, i.e. "fully expanded", pool) is shown in Figure 42, with the relationship obtained under steady state conditions (Figure 41) superimposed. This figure substantiates the strong correlation between the ARG pool and the level of arginase induction seen under "steady state" conditions (Figure 41).

However, there does appear to be a significant difference between the relationship observed under the "run down" as opposed to the "steady state" condition; for the same level of total ARG the level of arginase induction appears to be generally somewhat higher under the former compared with the latter condition. A hysteresis is indeed expected since under the "run down condition" preformed enzyme must decay before the specific activity determined in vitro reflects the lowered rate of synthesis in vivo in response to a fall in the signal, i.e. ARG pool. The hysteresis observed is however less than expected if arginase specific activity decays simply through growth dilution of enzyme. The more than exponential decay (with respect to ARG pool decay) argues that arginase enzyme is inherently unstable or actively degraded in vivo. (The enzyme is however quite stable in freeze dried mycelial powder where neither degradative mechanism can operate). It is also noticeable that the arginase specific activity starts to fall almost as soon as the ARG pool falls which argues that the time taken to translate a fall in signal level into a new and lower rate of enzyme synthesis is fairly short.

This hysteresis in arginase decay, albeit slight, must have an effect on the arginase flux at a given total extractable ARG pool. That the "steady state" and "run down" ARG to CIT relations are indistinguishable (Figure 34) shows that the

Figure 43: The "run down" relation between ORN and OTAase in arg-12 ure.

Data from Experiments Xb(∇) and IX (MIN, \star ; ARG, \star ;
ARG + ORN, \star) ,Tables 15 and 13, Appedix III.
See accompanying text for details.



corresponding to the time necessary to translate the change in signal into a change in synthetic rate. By a similar argument the synthetic rate would be expected to fall from maximal to minimal shortly after the transfer of cultures from ARG-supplemented to ARG-free medium through the rapid removal of the relatively small cytoplasmic pool.

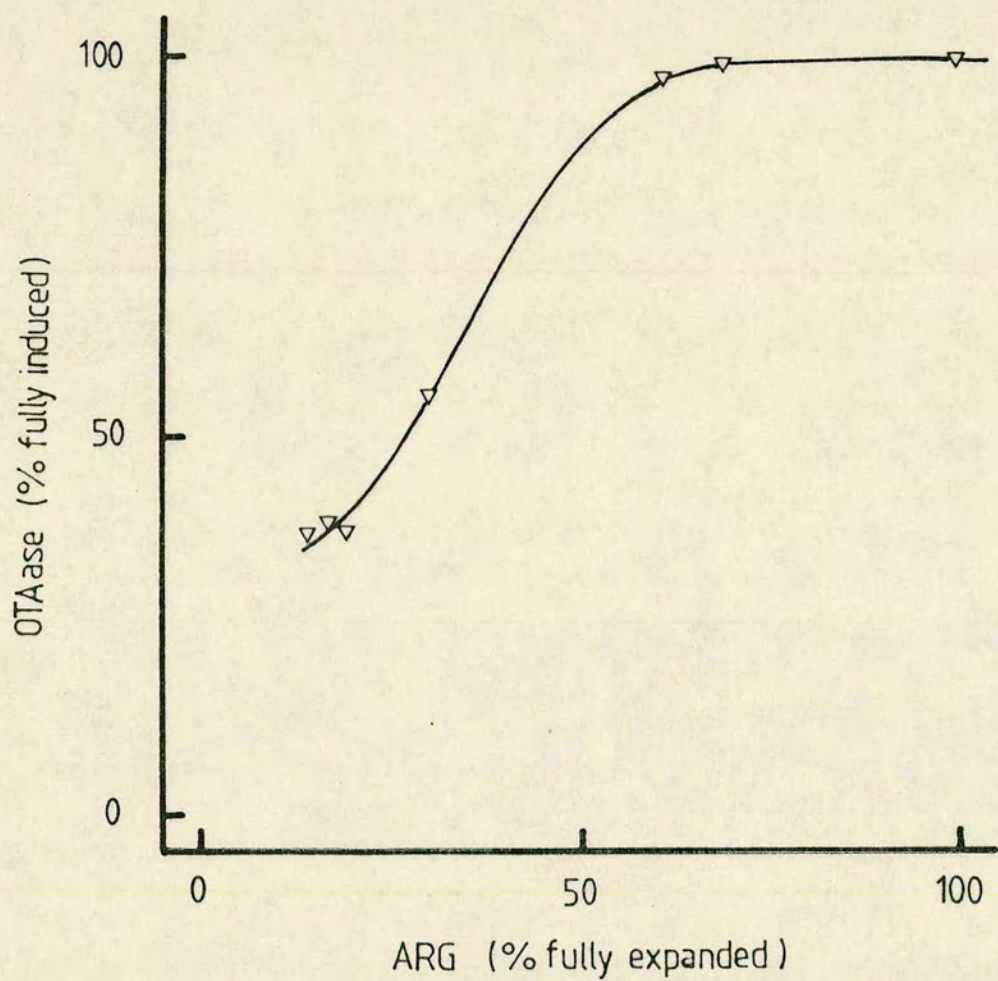
It follows that the apparent contradiction in behaviour of the induction process between the two studies is completely resolved by the differences in the experimental systems used. It has been tacitly assumed (Mainly from precedent and partly for mechanistic reasons) throughout this discussion that the coinducer is the cytosolic ARG pool or a direct derivative thereof. In fact the best evidence for this being the case comes from the observed behavioural difference between the two types of experimental system just discussed.

(11) OTAase

In the "run down" experiments, Xa and b, the specific activity of OTAase, like arginase, was observed to fall with time following the addition of HIS to the CIT-grown cultures (Figures 29 and 32). By analogy with the analysis of arginase modulation discussed above this is consistent with OTAase also being induced by ARG. However, as shown by Figure 43, an alternative interpretation of the data, as it stands, would be that the specific activity of OTAase is controlled by a derepression mechanism, the signal for which is the ORN pool. Because of the nature of the arg-12 ure experimental system the problem arises as to what is cause and what is effect. We have already discussed the relationship

Figure 44: The steady state relation between ARG and
OTAase in ure.

Data from Experiments VIIb and IX, Tables 11 and 13, Appendix III.
See accompanying text for details.

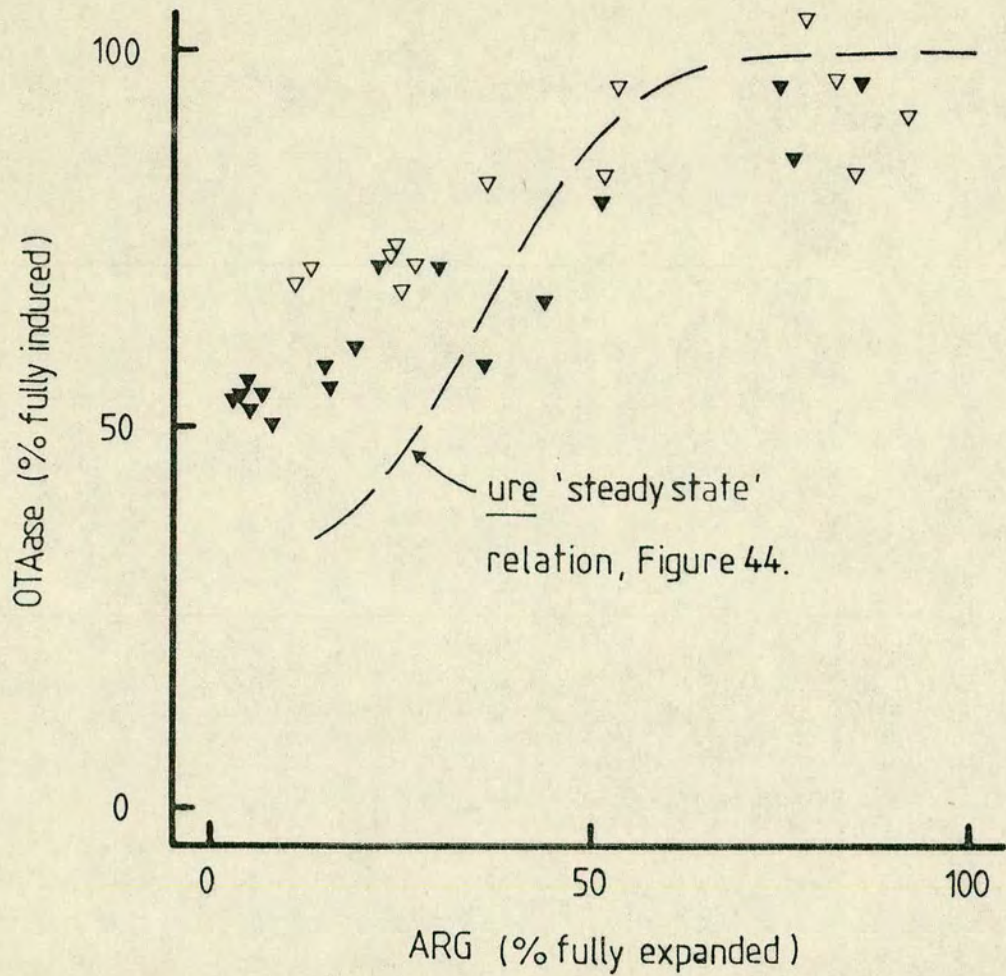


observed between the ARG and ORN pools under the same conditions (Figure 36) which we believe to arise principally through the effector role of ARG on the "proximal" part of the pathway. The derepression hypothesis arising from the plot of the "run down" data in Figure 43 is contradicted by a) the work of other investigators which argues that OTAase is induced by ARG (Castaneda et al., 1967 and Weiss and Anterasian, 1977) and b) is also refuted by the OTAase data from steady state ure cultures (Table 13) shown superimposed on the same Figure 43. It can be seen that the OTAase specific activity characteristic of ARG-grown ure cultures is about 2.5-fold higher than the MIN-grown activity despite almost identical ORN pools under both conditions. In the opposite way, both the ARG pools and the OTAase specific activities do not differ significantly between the ARG-grown and ARG + ORN-grown cultures despite the ORN pool being about 2.5-fold higher in the latter case.

As shall be seen, the data from both ure and arg-12 ure cultures under all conditions of growth are, however, consistent with OTAase being induced by ARG. This was established by performing the same type of analysis as described above for arginase. The mean level of OTAase induction for each steady state condition in ure is plotted against the corresponding, characteristic ARG pool in Figure 44 (data from Tables 11 and 13). The figure shows that, as for arginase, there is a strong positive correlation between the level of induction and the ARG pool level. Again, as drawn, the data can be interpreted as indicating an allosteric relationship between the signal and the enzyme. The

Figure 45 : The "run down" relation between ARG and
OTAase in arg-12 ure.

Data from Experiments Xa (▽) and b(▼), Tables 14 and 15,
Appendix III. See accompanying text for details.



overall degree of induction is, however, lower; the activity in CIT-grown cultures is only about 3-fold higher than in minimally grown cultures.

A comparable plot for the data from the "run down" experiments, Xa and b, is shown in Figures 45 with the relationship obtained under steady state conditions (Figure 44) superimposed. The figure agains shows a strong positive correlation between the ARG pool and the level of OTAase induction. However, under the "run down" condition the OTAase specific activity only falls to about 60% of the fully induced level for a fall in ARG pool to $2 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ whereas the specific activity characteristic of MIN-grown ure is about one-third of the fully induced level. This greater hysteresis compared with the arginase decay suggests that OTAase enzyme is inherently more stable and/or less prone to active degradation in vivo.

How then do the observations and interpretations made here with regards to the modulation of OTAase activity compare with those of other investigators? Weiss and Anteresian (1977) conducted a detailed study of the kinetics of OTAase induction following the addition of ARG to MIN-grown wild-type cultures and the kinetics of OTAase decay following the transfer of the induced cultures back to ARG-free medium. The system they used is thus identical to that of Wiess and Davis (1977), used to analyse arginase induction; the same problems therefore apply in the interpretation of their results and comparison with the present work.

Weiss and Anteresian (1977) established that induction requires both RNA and protein synthesis. They demonstrated that

inductive capacity was present within minutes of the addition of ARG to MIN-grown wild-type cultures, but that translation into induced enzyme synthesis required 30 to 40 min, essentially the same response time as observed by Weiss and Davis (1977) for arginase.

The conclusion from the present work that OTAase is stable in vivo receives direct support from Weiss and Anteressian (1977) who showed that the specific activity of OTAase remained substantially at the induced level following the inhibition of protein synthesis in ARG-grown cultures.

In addition they observed an almost immediate decay in OTAase activity at an exponential rate equal to the growth rate on transfer of induced cultures to ARG-free medium. From this they concluded that the rate of enzyme synthesis fell almost immediately on transfer from the fully induced rate to the basal rate characteristic of MIN-grown cultures and that the OTAase specific activity decayed simply by dilution through growth. This exponential decay occurred despite the presence of an extractable ARG pool still substantially larger than that characteristic of minimally grown cultures. This conclusion would appear to be at variance with the evidence presented here (Figures 44 and 45) for the level of induction being controlled by the level of the ARG pool. However, as with the arginase data of Weiss and Davis (1977) discussed earlier, this apparent disagreement with regards to the mechanism of induction can be resolved by consideration of the different nature of experimental systems used in the two studies; the almost immediate fall in synthetic rate to the basal level following the

complete removal of exogenous inducer is explained by the rapid removal of the small expanded cytosolic ARG pool, the true signal, and relatively slow egress of the large pool of vesicular ARG.

4.3.3.3

Synthesis, Summary and Conclusions

Synthesising the evidence from the foregoing analysis of the response of pools and enzymes to the inhibition of CIT uptake the following conclusions may be drawn with regards to the steady stateness of the system and the control of flux through the ARG pathway:

a) From the close agreement between the "steady state" and "run down" ARG versus CIT and ORN versus ARG pool relationships (Figures 22 and 34 respectively) we can conclude that under the "run down" condition the metabolic transformations are at a continually changing quasi-steady state throughout the post-HIS addition pool movements.

b) Comparison of the "run down" with the "steady state" relationships between the specific activity of the biosynthetic enzymes, AOGTase and ASAase, and the ARG pool (Figures 16 and 38 respectively) shows that the derepression system responds very rapidly to a fall in signal. Furthermore, the data suggests that under the "run down" condition the fall in the signal, i.e. the cytosolic ARG pool, leads to the fall in the total extractable ARG pool (Figure 40). This is believed to be due, at least in part, to the inhibition by HIS (and possibly also ORN) of ARG egress from the vesicle. However, no such effect is necessary for such a shift. A shift will arise if the rate of decay in flux from CIT to (cytosolic)

ARG is less than the normal rate of vesicular equilibration.

c) As the ARG pool is caused to fall into the "derepression range", primarily by the inhibition of CIT uptake, so derepression itself tends to buffer the pool from further change. In Experiment Xb the ARG pool is "held" at a value of about $1 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$, around the steepest part of the derepression curve. To achieve any further decline in the ARG pool and increase in the level of derepression would require a very small, further reduction in the rate of CIT uptake. This is because as the rate of CIT uptake is progressively reduced so the Sensitivity Coefficient of the step increases from a very low value ("no control") to approach unity ("controlling"). On the linear part of the curve a very small change in the rate of CIT uptake will elicit a large change in flux to ARG (see Figure 6 for the relation between the amount of enzyme and the pathway flux). Technically the attainment of lower ARG pools and higher degrees of derepression would be difficult by this method since at these values the proportion of the flux to ARG accounted for by the expansion of the ARG pool itself is very small.

d) The level of induction of both OTAase and arginase is related by an allosteric function to the total extractable steady state ARG pool (Figures 41 and 44). This is consistent with the binding and dissociation of the coinducer and the inducer being an allosteric transformation (Burns and Kacser, 1977). Although limited, the data do suggest that there is a significant difference in the ARG pool function between the two enzymes. However, no statement can be made as to the stage(s) in the overall induction

induction process(es) at which the difference(s) reside(s).

Under the "run down" condition there is a significant hysteresis compared with the steady state condition in the decay of both arginase and OTAase (Figures 44 and 45). This is due to the relative stability of the preformed enzymes in vivo compared with the rate of change in the ARG pool. This effect is quite substantial for OTAase but quite small for arginase. In minimally grown ure the specific activity of OTAase is about 30% and arginase about 15% of the CIT-grown value (Figures 44 and 45 respectively). For the same fall in total ARG pool under the "run down" condition the specific activity of OTAase only falls to about 60% and arginase to between 30 and 40% of the CIT-alone level (Figures 41 and 42 respectively).

That the CIT to ARG and ARG to ORN relations are virtually indistinguishable between the "steady state" and "run down" conditions despite the lag in arginase decay argues that the consequential difference in arginase flux is ^arelatively small component of the total flux out of ARG.

The lag in arginase decay under the "run down" compared with the "steady state" condition cannot account for the more pronounced shift from the bradytrophic derepression curve to higher values of total extractable ARG under the former condition. As will be shown later in the text, no arginase flux can be detected at these levels of ARG. Furthermore, we shall see that a similar shift in the curve is observed where the arginase flux is mutationally lacking (Section 4.3.7.2).

e) The non-linear relationship between ARG and CIT observed under all experimental conditions in arg-12 ure (Figures 22,23 and 34)

is best explained by the flux to ARG becoming progressively saturated at level of CIT above about $5 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ There is no evidence for a change in either the amount or activity of the ARG forming enzymes over this range (Davis *et al.*, 1978a). The observed variation in arginase specific activity (Figures 41 and 42) will affect but cannot generate the relationship. The limited data on ASA pools and the resultant CIT to ASA and ASA to ARG relations (Flint, Porteous and Kacser, in preparation) suggests that saturation of SYNase at these levels of CIT accounts for most of the non-linearity in the CIT to ARG relation although there is also evidence for some saturation of ASAase at high substrate levels.

f) The ARG-mediated variation in the specific activity of OTAase (Figure 44) must have a positive effect on the flux from ORN to GSA. However, the evidence from the PRO responses (Figure 37) indicates that under our conditions the effect of induction on the OTAase flux is much less than the mass action effect of accumulated ORN. This implies that the concentration of ORN which OTAase "sees" varies around the K_m for the enzyme. This is in complete accord with the observations that OTAase is localised to the cytosol (Weiss and Davis, 1973) and that in minimally grown wild-type *Neurospora* less than 5% of the total extractable ORN is cytosolic with a concentration around the K_m for the enzyme determined *in vitro* (Karlin *et al.*, 1976). The compartmentation of ORN and the subcellular localisation of the pathway enzymes were described and discussed at some length in Section 1.3.2.1 to this thesis.

g) A dramatically non-linear, inverse relationship between ORN and ARG is observed in arg-12 ure under all experimental conditions

(Figures 24, 25 and 36). The ORN response to ARG can be divided into three parts. Firstly, the ORN pool is substantially constant, with a mean of $0.4 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ in Experiments Xa and b, at ARG values from fully expanded down to about $5 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ This occurs despite the fact that the catabolic flux to ORN must be falling due to a) the deinduction of arginase (Figure 42) and b) the mass action effect of falling substrate. Flint (1977) showed that the rate of urea accumulation, equivalent to the catabolic flux to ORN, was proportional to the ARG pool. On CIT supplement alone this flux is greater than the flux to expansion of the ARG pool itself but in ure on minimal medium falls to only about a quarter of the ARG expansion flux, equivalent to less than 5% of the total flux out of ARG. The same conclusion can be drawn from a manipulation of data from the present work to be described later in the text (see Section 4.3.7.3). The work of Davis and colleagues (Karlin et al., 1976; Bowman and Davis, 1977a and b) indicates that ARG derived ORN is predominantly catabolised further by OTAase. However, the PRO pool is also essentially constant, with a mean value of $0.44 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ in Experiment Xa and b, over this same range of ARG pools. No movement of any other metabolically related pool is observed, which could account for this "lost" flux. Several formal explanations for the lack of ORN and PRO response can be constructed. Firstly, the inferred decrease in catabolic flux may be oppositely balanced by an ARG-mediated increase in biosynthetic flux. Secondly, the ORN pool may be "locked" by the feedback inhibition of ORN on its own synthesis. Thirdly, ORN biosynthesis may be reversible to an extent. Finally, the possibility must be considered that the

OTAase flux over this range of ARG pools is in fact substantial but "lost" in GLU. In view of the central role of this metabolite in intermediary metabolism a considerable amount of buffering would be expected against changes in rate of formation. A large increase in flux to GLU from ORN via OTAase could be "lost" as many small changes in rates of formation and utilization of GLU by other pathways. It will be shown later in the text that this last alternative is in fact the real explanation for the lack of ORN and PRO response (see Section 4.3.7.1).

No mention has yet been made of the remaining, known fate of ORN, namely via ODCase to polyamine synthesis. We have no direct estimate of this flux under our conditions and must rely on the evidence of Davis and coworkers (Karlin *et al.*, 1976) that this flux remains substantially constant under conditions of varying ARG and ORN pools; a flux of $2.2 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ increase was calculated.

The second phase of the ORN response is that part of the ORN rise seen as the ARG pool falls below $5 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ but before the derepression range is entered. In this phase the ORN pool expands about four-fold. Furthermore, as soon as the ORN pool increases a proportional increase in the PRO pool is observed (Figure 37). In accord with the observations of Flint (1977), the change in arginase flux over this range of ARG pools can be shown to be very small (see Section 4.3.7.3). The ORN and PRO pool response must therefore reflect an ARG-mediated increase in biosynthetic flux affected through the deinhibition of one or more enzymes in the "proximal" part of the pathway.

The third and final phase of the ORN pool response covers the rise in ORN seen once the ARG pool falls to within the derepression range. Barthelmess et al (1974) have shown that the last three enzymes of the ARG pathway are coordinately derepressed. From the evidence presented here (Figure 39) this coordinancy probably extends to all of the enzymes in the "proximal" part. Of all the pathway enzymes there is only evidence for CPSase-ARG, an enzyme "beyond" ORN, being outwith this control (Cybis and Davis, 1975). Assuming that derepression is coordinate throughout the "proximal" set of enzymes the 2-fold increase in AOGTase specific activity observed in Experiment Xb in this last phase must lead to an equal increase in the flux to ORN. We can estimate the change in flux from the ORN and PRO pools extrapolated from Figures 36 and 37 (assuming an ARG pool of $2.49 \mu\text{mol. } 100^{-1} \text{ mg d.wt.}$ as the minimum fully repressed condition (mean value for ure on MIN from Tables 11 and 13) and $0.8 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ as the ARG pool at 2-fold derepression (Figure 38)) as follows:

$$\begin{aligned} \text{"Repressed" flux to ORN} &= F \times \text{ORN} + F \text{ ODCase} + F \text{ OTAase} \\ &1.30 + 2.2 + (0.95 - 0.31) \\ &4.14 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. increase} \end{aligned}$$

$$\begin{aligned} \text{"Derepressed" flux to ORN} &= F \times \text{ORN} + F \text{ ODCase} + F \text{ OTAase} \\ &4.5 + 2.2 + (1.8 - 0.31) \\ &8.19 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. increase} \end{aligned}$$

Following through the reasonable assumption of coordinate derepression this calculation suggests that in this range of ARG pools derepression is the only mechanism which is operating to change the biosynthetic flux. However, this conclusion relies

on the assumption that the increase in the PRO pool accurately reflects the OTAase flux, an assumption which as already discussed, is open to doubt. The question of the range and extent to which deINH and derepression operate to control the flux to ORN will be fully answered in Section 4.3.7.3.

4.3.4

Experiment XI : the "run up" condition in *arg-12* ure

In Experiments Xa and b (Section 4.3.3) we established, by comparison with "steady state" experiments, that under the "run down" condition the system as a whole was at a metabolic quasi-steady state throughout the post-HIS addition pool movements. These experiments also confirmed and elaborated upon the effector role of ARG in the control of the amount and activity of the pathway enzymes and hence the fluxes through the pathway. Again by comparison with the "steady state" condition, we established that the derepression system responded with a new elevated rate of enzyme synthesis with almost no lag as the ARG pool fell. In contrast we observed a significant lag in the decay of induced catabolic enzyme under the "run down" condition. This was presumably due to the stability of preformed enzyme since we believe that the time taken for the translation of a change in signal level into a change in synthetic rate to be relatively short. The possible effects of such delays in enzyme adjustments on pool and subpool movements and metabolic fluxes have been discussed.

As a result of the foregoing analyses we might well expect the effect of a change in the "controlling" component, i.e. the ARG pool on the response of a "controlled" component, e.g. the amount of enzyme activity to be dependent not only on the rate

but also the direction of change of the "controlling" component.

It was therefore felt to be of some considerable interest to examine the possibility of conducting an experiment essentially in reverse to those just described. That is to say, to start, at the beginning of the experimental time course, with an ARG pool well within the derepression range and with the consequent and characteristic levels of related metabolites and pathway enzyme specific activities and to monitor the changes in all these components following an experimentally controlled increase in the ARG pool over time. With this aim in mind the following experiment detailed in Table 16 was conducted.

Thirteen growth flasks were prepared and treated as if for a "run down" experiment with HIS added about 13 h after inoculation into CIT supplemented medium. After a further 8 h incubation (by which time the ARG pool would be well within the derepression range, as judged by Experiment Xb (Figure 31)) more CIT was added to each of nine flasks to change the medium HIS:CIT ratio from 5:1 to essentially 1:1. These nine flasks were harvested sequentially over approximately 5 h following the addition of more CIT to the medium and with roughly equally intervening time intervals. Of the four flasks to which no more CIT was added two were harvested before and two after the time of the addition of more CIT to the other nine flasks. Full experimental details and the results of the various analyses performed are shown in Table 16, from which the following observations can be made and conclusions drawn:

(1) Growth rate

The log of the dry weight of mycelial harvest yields are

Figure 46: Experiment XI: growth rate.

Data from Table 16, Appendix III.

See accompanying text for details.

KEY

Symbol :- ● □
Flask no. :- CH1 to 4 CH+C 1 to 9

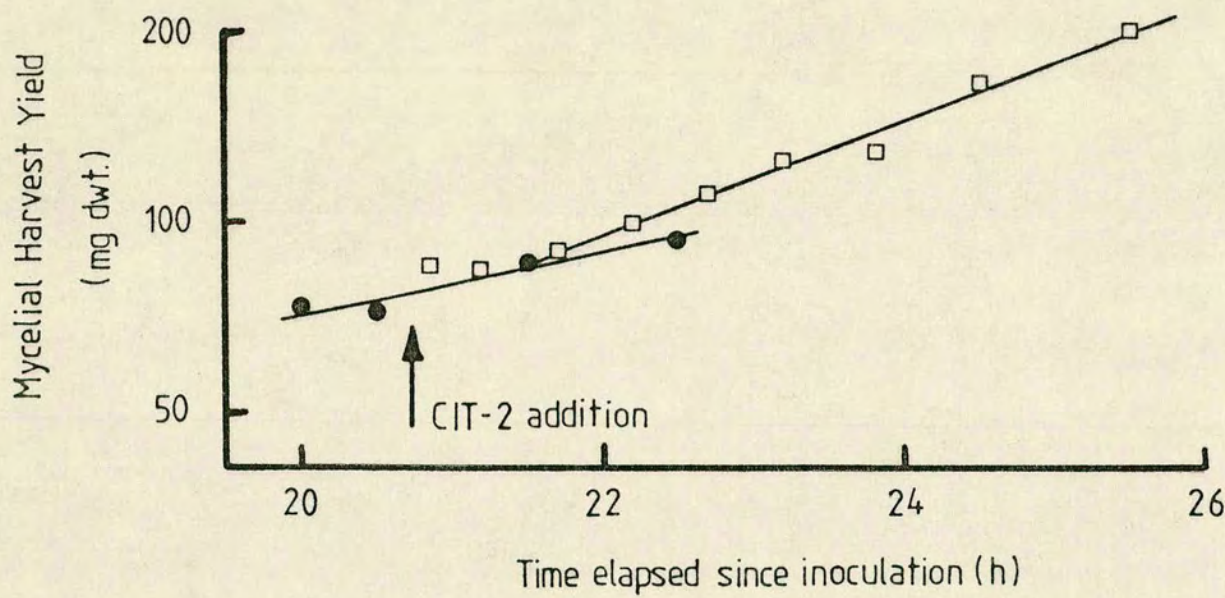
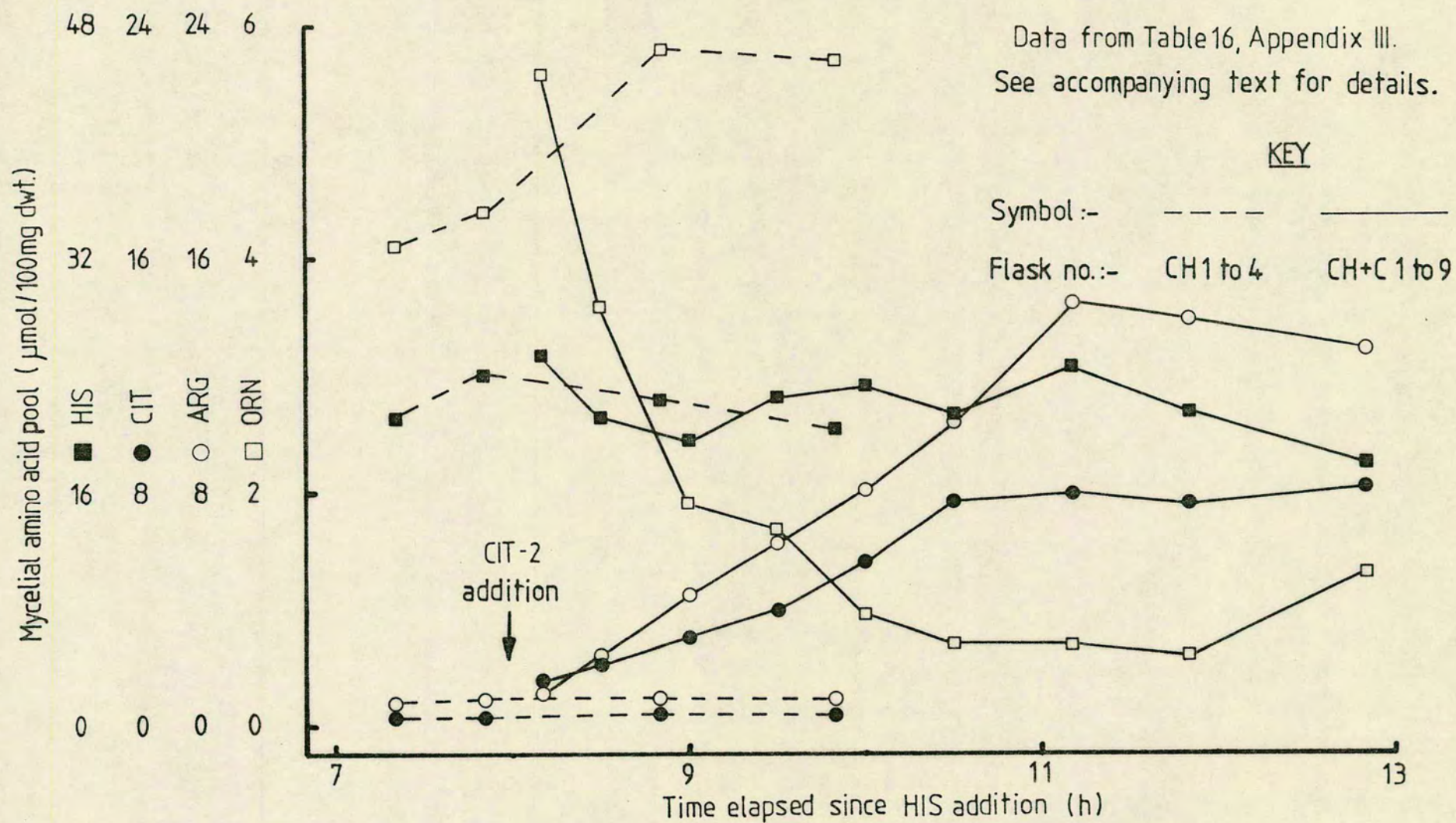


Figure 47: Experiment XI: the post-CIT2 addition changes in mycelial amino acid pools.



plotted against the time elapsed since inoculation in Figure 46. The figure suggests that the growth rate of the cultures slowed significantly following the addition of HIS to the cultures compared with that normally observed for cultures grown in medium supplemented only with CIT (Figure 13). Extrapolation of the growth curve formed by the harvest yields from the four cultures grown solely on CIT plus HIS (CH 1 to 4) medium gives a doubling time of approximately 6 h, twice that of cultures grown solely on CIT supplemented medium. This growth rate effect is understandable in view of the earlier time of HIS addition employed here compared with the "run down" experiments Xa and b (see Tables 14 and 15). The effect of the time of HIS addition on the growth rate has already been discussed (see Section 4.1.6). After about one hour's incubation rapid exponential growth with a doubling time of 3 h 20 min was restored to those cultures to which more CIT was added.

(ii) Mycelial aminoacid pools

The complete data on mycelial aminoacid pool levels in the cultures are presented in Table 16. The mycelial HIS, CIT, ARG and ORN are plotted against time elapsed since HIS addition to the medium in Figure 47, from which the following observations can be made and conclusions drawn:

a) In those cultures grown only on CIT plus HIS supplemented medium (CH1 to 4) the pools are substantially time invariant with the exception of ORN which shows a significant increase with time. As expected, the absolute pool values closely match those observed in Experiment Xb (Table 15) at equivalent times elapsed since HIS addition thus further substantiating the belief that the experimental

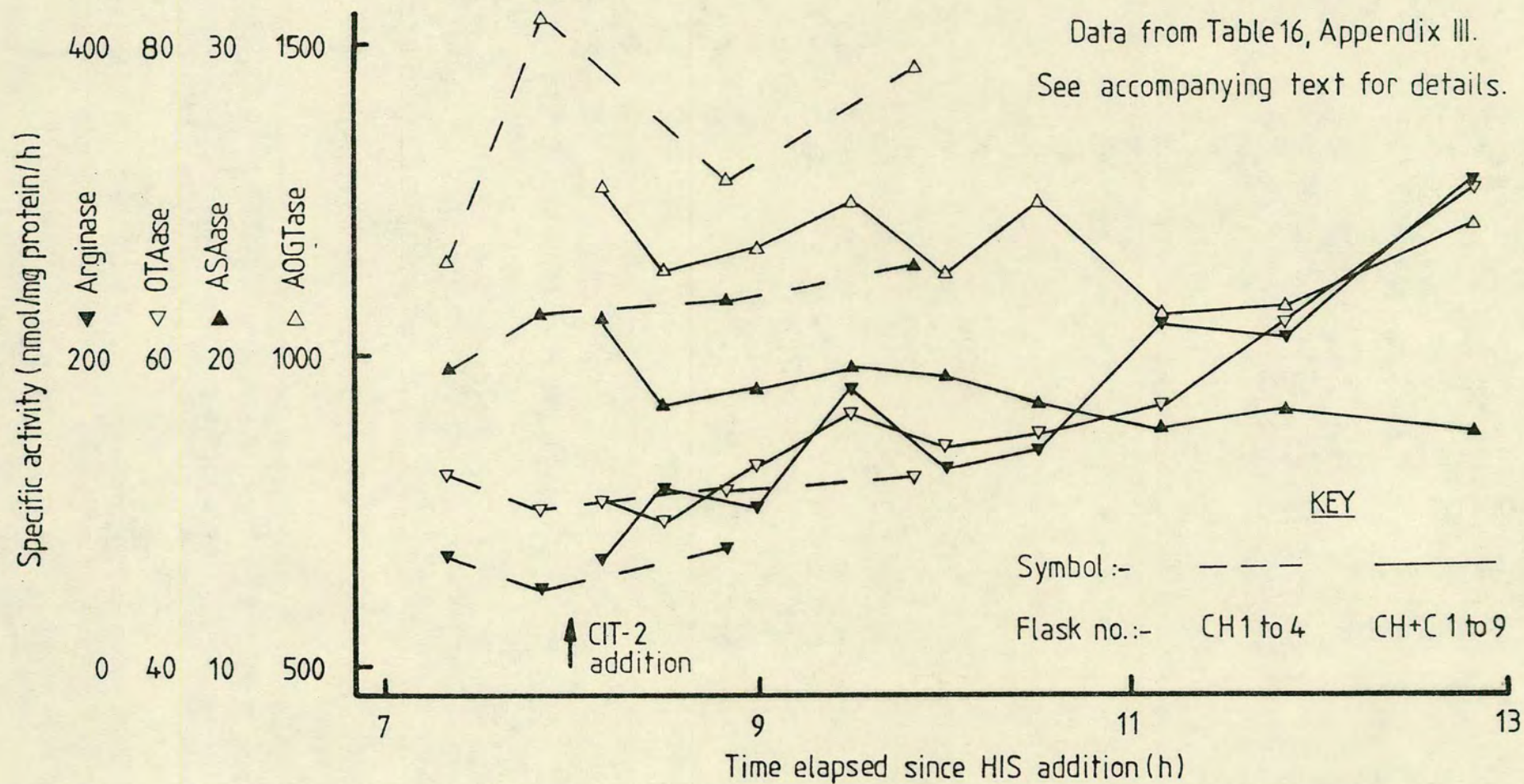
methodology is reliable, repeatable and predictable.

b) The CIT pool increases rapidly immediately following the addition of more CIT to the medium reaching a new steady state level of about $8 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ after $2\frac{1}{2}$ h. The overall pool expansion is about 20-fold. The steady state level reached is considerably less than that characteristic of cultures grown on CIT only, reflecting the continued but reduced inhibition of CIT uptake by HIS.

c) In contrast to the CIT pool, no expansion of the ARG pool is detectable in the first 10 min period following the addition of more CIT to the medium. Thereafter the ARG concentration increases steadily, settling out at about $14 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ some 3 h after the addition of more CIT to the medium (and about 40 min after the CIT pool itself had reached a steady state level). The comparative time courses of CIT and ARG pool expansion must generate a different relationship between these two pools from that described by the "steady state" and "run down" experiments (Figure 22 and 34 respectively). Comparison of the relationships should help to identify the elements of importance in the control of flux from CIT to ARG. The exact form of the "run up" relationship and the conclusions which may be drawn from it with respect to the control of flux will be discussed in Section 4.3.5.

d) The most dramatic post-CIT addition response is seen in the ORN pool which crashes almost as soon as an increase in the ARG pool is observed. This is consistent with the allosteric inhibition of one or more enzymes in the "proximal" part over a change in the ARG pool from about 1 to $2 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ This relatively

Figure 48: Experiment XI: the post-CIT2 addition changes in enzyme specific activities.



small change in the total extractable pool may hide a considerable expansion of the cytosolic pool. The question of the control of flux in the "proximal" part will be returned to in Section 4.3.5.

e) The marginal fall in the HIS pool over the experimental time course contrasts with the rapid increase in the mycelial CIT pool following the addition of more CIT to the medium. This implies that the pre- and post-CIT addition rates of HIS uptake are much the same, consistent with HIS being predominantly transported by an uptake system which is not inhibitable by CIT, i.e. the "basic" or "neutral" (Pall, 1970a and b; see Appendix II to this thesis).

(iii) Enzyme specific activities

The specific activity of each of the four enzymes, OTAase, arginase, AOGTase and ASAase, was determined in all of the experimental cultures. The data are presented in Table 16, and plotted against the time elapsed since HIS addition in Figure 48, from which the following observations can be made and conclusions drawn:

a) The specific activities of both catabolic enzymes, OTAase and arginase, are low in each of the four cultures to which no second CIT addition was made. This is as expected given the corresponding ARG pool and the findings of Experiment Kb (Figure 31).

Both activities increase coordinately with time following the addition of more CIT to the other nine cultures. The specific activity of the two enzymes increase slowly to begin with increasing in rate towards the end of the experimental time course and, significantly, beyond the point at which the ARG pool had settled to its new steady state value. The induction of these two enzymes

will be discussed in Section 4.3.5.

b) The specific activities of both biosynthetic enzymes, AOGTase and ASAase, are derepressed in each of the four cultures to which no second CIT addition was made. Again this is as expected given the corresponding ARG pool and the findings of Experiment Xb (Figure 32).

Both activities fall coordinately with time following the addition of more CIT to the other nine cultures. However, the rate of fall is very slow which suggests that not only are both enzymes stable in vivo but also that the time taken for an increase in signal (i.e. ARG pool) to be translated into a repressed rate of enzyme synthesis may be much longer than the time taken to respond with a derepressed rate of synthesis to a falling ARG pool. This question will be discussed further in Section 4.3.5.

Clearly the marginal decline in the specific activity of AOGTase cannot account for the dramatic fall in the ORN pool which must, as previously stated, reflect the response to a change in activity rather than amount of enzyme.

In summary, it is clear from a comparison of the time course of pool and enzyme changes observed in this "run up" experiment with those observed under the "run down" condition that the relationships between system components are indeed affected by the direction as well as the rate of change of the components. In the next section these hystereses will be discussed and interpreted with respect to the control of flux through the pathway.

4.3.5 Comparison of the "run up" with the "run down" condition in
arg-12 ure

4.3.5.1 The relation between the mycelial ARG pool and the specific
activity of the pathway enzymes

(1) The biosynthetic enzymes

It was concluded from the marginal decline in the specific activities of both AOGTase and ASAase in the "run up" experiment (Figure 48) that both enzymes were quite stable in vivo. It was also suggested that there may be a significant hysteresis in the response to a change in ARG from "derepressing" to "repressing" levels compared with the rapid response to change observed in the opposite direction (Figure 3). This question will be pursued in the following calculation:

The rate of fall in ASAase activity (Figure 48, slope estimated by eye) is approximately $1 \text{ nmol. mg protein}^{-1} \cdot \text{min}^{-1}$ per mycelial doubling time (3 h 20 min). By 30 min after the second addition of CIT (culture CH + C2) the ARG pool has risen to significantly above $2.0 \text{ } \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$, a level at which the level of ASAase activity is normally fully repressed. Taking as the fully repressed level of ASAase synthesis $12.2 \text{ nmol. mg protein}^{-1} \cdot \text{min}^{-1}$ (Table 15), the level of ASAase specific activity would be expected to have fallen by approximately $4 \text{ nmol. mg protein}^{-1} \cdot \text{min}^{-1}$ within one doubling time simply by dilution. This assumes that a) the response in the rate of enzyme synthesis to a change in the "signal" pool level was immediate, and b) that the enzyme was stable in vivo. If the enzyme were not stable the fall in specific activity would be expected to be even greater. That the ASAase specific

activity falls by as little as $1 \text{ nmol.mg.protein}^{-1} . \text{min}^{-1}$ suggests that the synthesis of ASAase continues at a derepressed rate long after the total ARG pool has expanded to "repressing" levels.

Similarly the "expected" fall in the AOGTase activity in the first mycelial doubling after the total ARG pool has expanded to "repressing" levels would be about $300 \text{ nmol. mg protein}^{-1} . \text{min}^{-1}$. However, the observed rate of decay is only one-third of the "expected".

This asymmetry in response with regard to the direction of the change in the "signal" pool might be explained if the equilibrium between unbound and bound corepressor strongly favours the dissociation of corepressor from the repressor molecule. The persistence of derepressed enzyme levels despite a repressing level of ARG differs from the "cross pathway" regulation described by Carsiotis et al (1970) and discussed in Appendix I to this thesis, in that here we are resupplying the same corepressor originally reduced to a derepressing level. Certainly, as will be demonstrated, the persisting derepressed levels of enzymes cannot be explained by the cytosolic ARG pool persisting at a "derepressing" level despite an increase in the total extractable pool. Whatever the cause of the hysteresis the effect must be to increase proportionally the flux to ARG for a given concentration of CIT compared with the repressed condition.

(ii) The catabolic enzymes

a) Arginase

The arginase induction system appears to respond fairly rapidly in the "run up" experiment with about a four-fold increase in specific activity for an increase in ARG from 1 to 10 $\mu\text{mol. } 100 \text{ mg}^{-1}$ d.wt. (Figures 47 and 48). Much the same change in specific activity for an equivalent pool change was observed under the "steady state" condition (Figure 41). The rapid response is in accord with the findings of Weiss and Davis (1977) discussed above in Section 4.3.3.2 and implies that the cytosolic ARG pool expands almost as soon as more CIT is added to the medium.

b) OTAase

In contrast with the arginase response, the OTAase induction system appears to be very slow to respond to the increase in the ARG pool; only a marginal increase in specific activity is observed under the "run up" condition (Figure 48) compared with the change in specific activity observed under "steady state" conditions (Figure 44), for equivalent changes in the ARG pool. This substantiates the inference made from differences in the behaviour of OTAase and arginase under the "run down" condition (Section 4.3.3.3) that the induction systems for the two enzymes are fundamentally different. At what level this difference resides is not known.

Figure 49: The "run up" relation between CIT and ARG in arg-12 ure.

Data from Table 16, Appendix III.

See accompanying text for details.

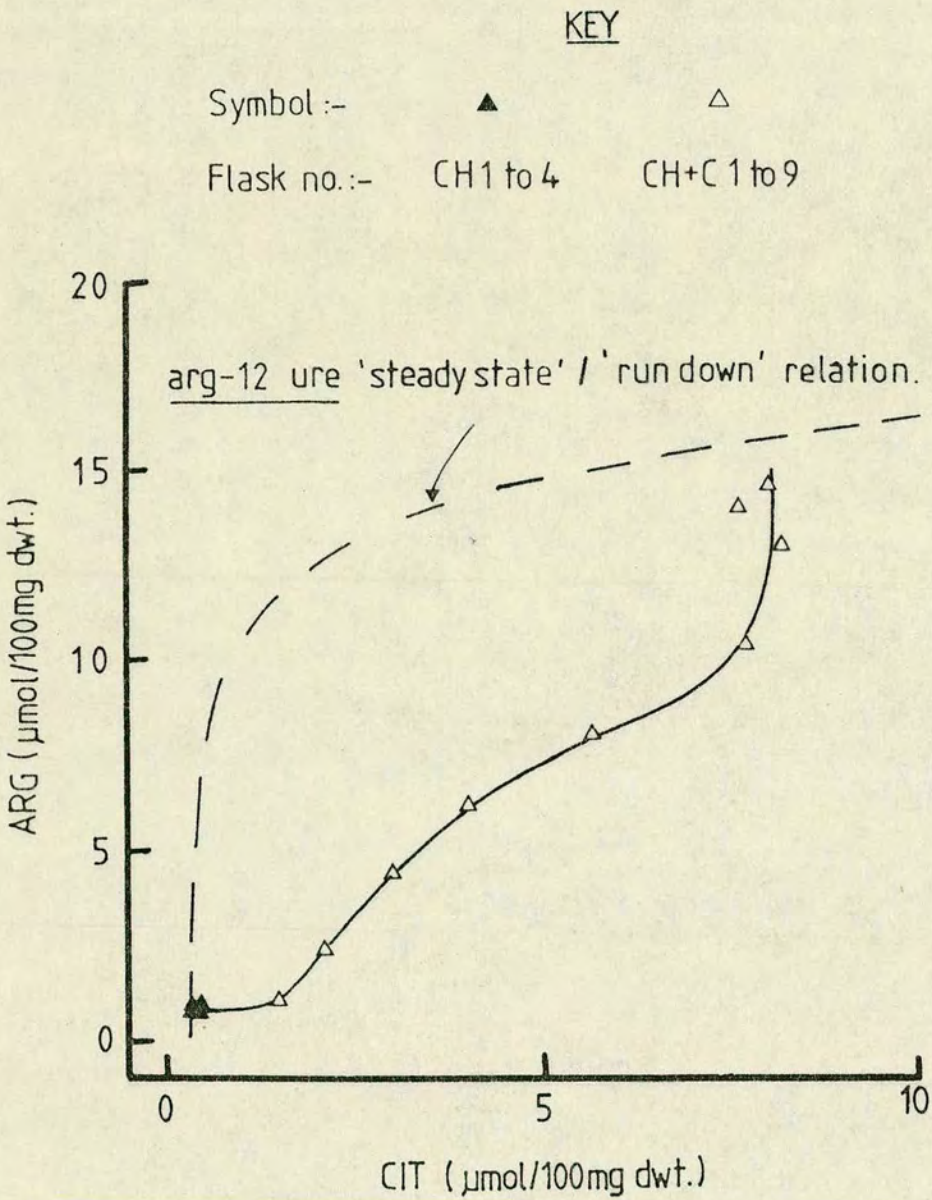


Figure 50: The "run down" relation between CIT and FxARG in arg-12 ure.

Data from Experiments Xa (○) and b (●), Tables 14 and 15, Appedix III.

See accompanying text for details.

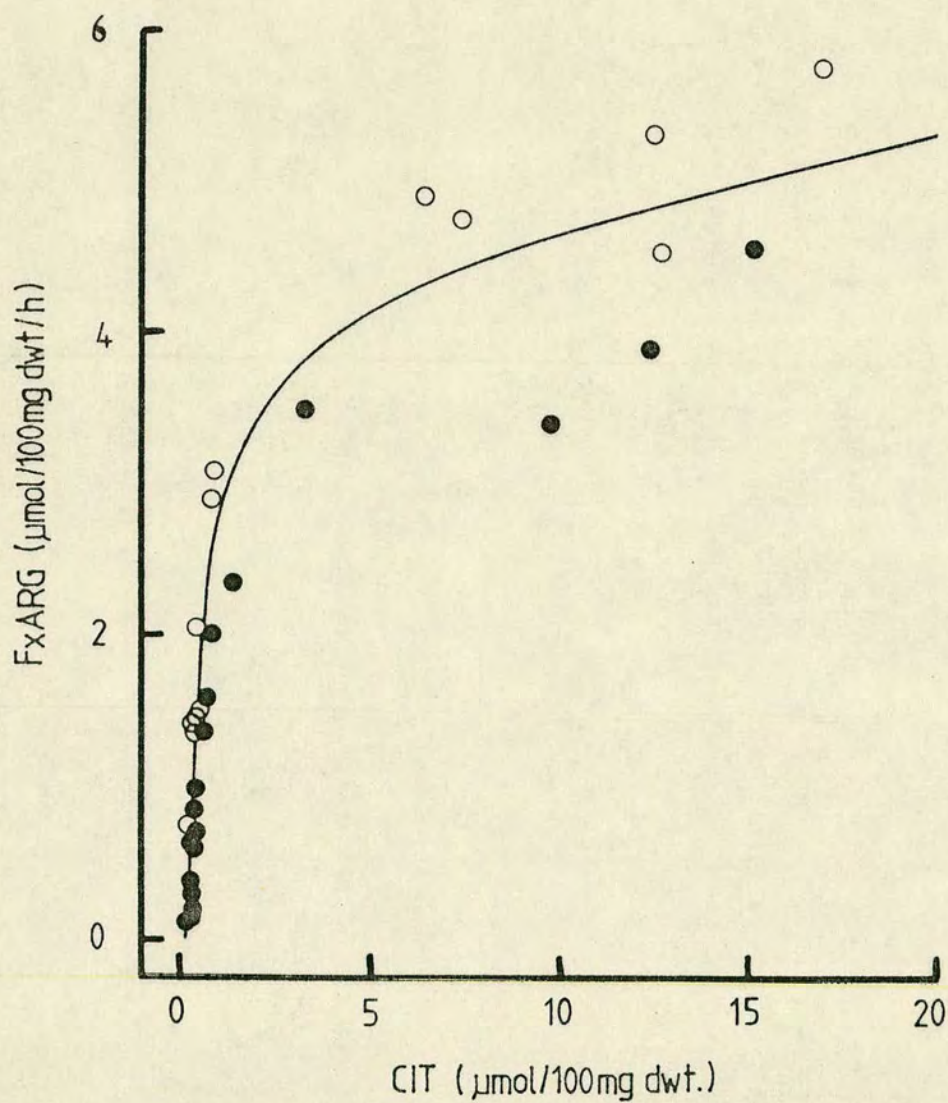


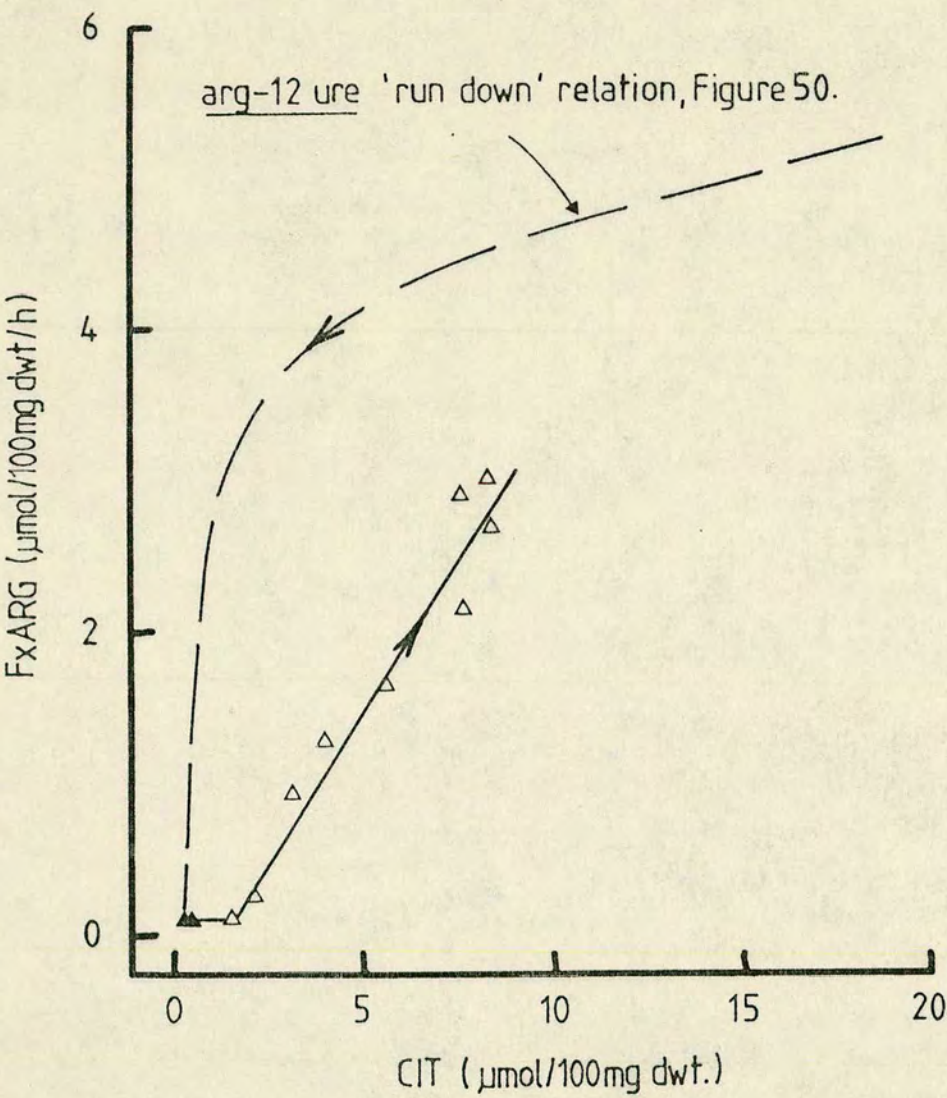
Figure 51: The "run up" relation between CIT and FxARG in arg-12 ure.

Data from Table 16, Appendix III.

See accompanying text for details.

KEY

Symbol :-	▲	△
Flask no. :-	CH1 to 4	CH+C 1 to 9



4.3.5.2

Correlations between aminoacid pools

It was apparent simply from the visual inspection of the relative changes in the CIT and ARG pool in Experiment XI (Figure 47) that under the "run up" condition the relationship between these pools must differ substantially from that described both by "steady state" cultures (Figure 22) and the "run down" condition (Figure 34). The extent to which this is so is seen in Figure 49 where the data from Experiment XI are plotted, connected by a solid line, and where the dashed line represents the "steady state"/"run down" relation. Clearly the ARG pool responds to an increase in CIT influx in a substantially different way from the response to a decrease in CIT influx. However, particularly in view of the growth rate effects in Experiment XI (Figure 46), the more appropriate plot for comparison of the different experiments is the relation between the CIT pool and the ARG concentration per unit growth, i.e. the ARG expansion flux. The data from the "run down" experiments Xa and b (Tables 14 and 15) are plotted in this manner in Figure 50. The corresponding plot for the "run up" experiment XI (Table 16) is shown in Figure 51 with the relationship derived from Figure 50 superimposed.

The most striking feature of the "run up" condition is the directly proportional increase in the ARG expansion flux with increasing CIT, but at a rate considerably lower than the corresponding fall in ARG with CIT observed under the "run down" condition. This deviation from the "run down" or "steady state" behaviour is particularly surprising in view of the substantially derepressed levels of ASAase (and, by inference, SYNase also) observed throughout the time course of Experiment XI (Figure 48).

This higher concentration of enzyme must result in a proportional increase in the flux from CIT to ARG at equivalent CIT pools compared with the repressed condition which prevails over all but the tail end of the "run down" condition (Figure 32). Since no evidence has been found for the activity of either SYNase or ASAase being affected by activation or inhibition in vitro or in vivo (Davis et al, 1978a) the disparity between the ARG to CIT relations can only be explained by the flux out of ARG being that much greater for a given flux into ARG under the "run up" compared with the "run down" or "steady state" condition. More specifically, the difference between the "run up" and "run down" relation must reside in disparate rates of hydrolysis by arginase since the flux of ARG to protein has been shown to be essentially constant under all exponential growth conditions (Tateson, 1971 and Flint, 1977).

What differences in experimental conditions might explain this unexpected behaviour? The flux through arginase will be affected by three factors:

- i) the amount of enzyme present, i.e. the level of induction by ARG.
- ii) the enzyme activity, which is feed back inhibited by ORN (Mora et al, 1972 and Davis et al, 1978a), and
- iii) the substrate concentration, i.e. the cytosolic ARG pool.

The K_m for native arginase determined in vitro is of the same order as the cytosolic ARG concentration in minimally grown wild-type Neurospora (Weiss, 1973 and Subramanian et al, 1973). This explains why the rate of urea production is very low under such conditions despite substantial enzyme activity in vitro and a high total

extractable ARG pool (Tateson, 1971).

The change in arginase specific activity in response to a change in ARG pool is of the same order under both the "run up" and "run down" conditions (Figures 49 and 42 respectively) and therefore the first factor cannot account for the difference in the ARG to CIT relation. However, two additional and striking differences in pool movements are observed which suggest that the second and third factors operate to different degrees between the "run up" and "run down" experiments and can explain the assumed disparity in arginase fluxes.

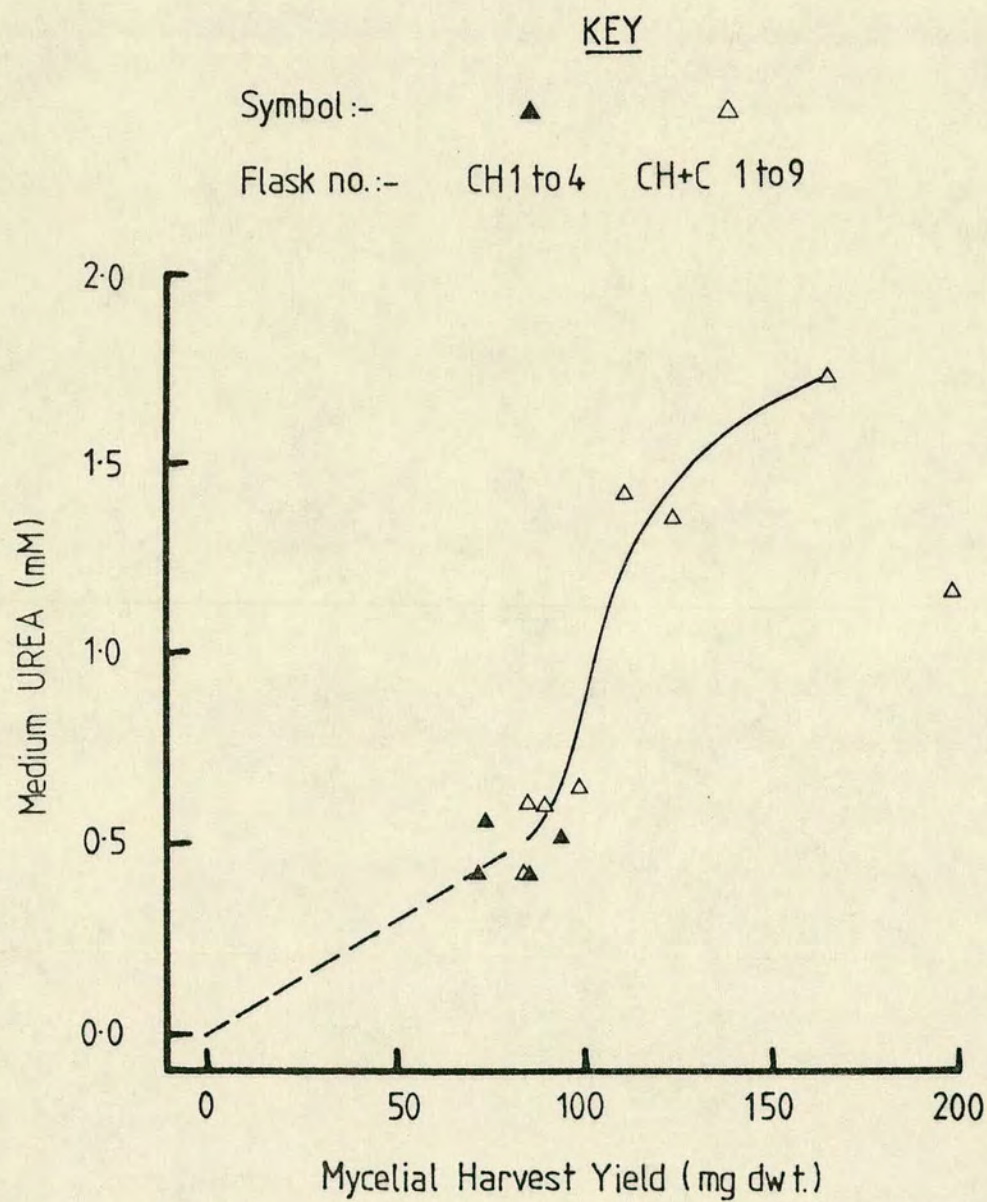
Firstly, by nature of the experimental design, we start with a low mycelial HIS pool in the "run down" experiments which rises with time to reach a high steady state level after one or two mycelial doublings (Figures 26 and 31). In contrast, the mycelial HIS pool and the rate of HIS uptake starts and remains high in the "run up" experiment (Figure 47).

Secondly, in the "run down" experiments the ORN pool is initially low and only increases significantly after the ARG pool has fallen to below about $5 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ (Figure 36) whereas the ORN pool starts high and crashes as soon as expansion of the ARG pool is detected in the "run up" experiment (Figure 47).

Extending the argument given in Section 4.3.3.2 to explain the shift in the derepression curve under the "run down" condition to high total extractable ARG pools, the effect of the high mycelial HIS and ORN will be to inhibit the uptake of cytosolic ARG under the "run up" condition. Together with the derepressed flux from CIT to ARG, the net effect will be the rapid expansion

Figure 52: Experiment XI: the rate of medium urea accumulation.

Data from Table 16, Appendix III.
See accompanying text for details.



of the cytosolic ARG pool to a level much higher than the "steady state", or "run down" level, corresponding to the observed total extractable pool. Mass action will result in a high arginase flux despite an initially low level of enzyme specific activity. Furthermore as the ORN pool crashes so the inhibitory effect of ORN on arginase will fall.

The expectation of an elevation in the arginase flux immediately following CIT-2 addition in the "run up" experiment was tested by determining the rate of urea formation before and after the addition of more CIT to the medium. The concentration of urea present in the medium samples taken at the time of harvesting was estimated by the BUN method. These concentrations are plotted against the corresponding harvest yields in Figure 52 from which it can be seen that the arginase flux must indeed have been considerably lower before than after the addition of more CIT to the medium. In fact, the plot shown in Figure 52 underestimates the real difference in rates of urea accumulation before and after the addition of more CIT for two reasons. Firstly, the rate of urea accumulation would have been initially very high but fallen progressively with time following the addition of HIS to the medium (Flint, 1977). Secondly, although most of the urea formed is excreted into the medium where it accumulates, a significant steady state pool is held in the mycelium which will of course constitute an exponentially increasing total amount with time (Flint, 1977).

We can now turn specifically to the comparison of the ORN to ARG pool relation between the "run up" and "run down" condition, bearing in mind the interpretation of the system's behaviour from

Figure 53: The "run up" relation between ARG and ORN in arg-12 ure.

Data from Table 16, Appendix III.

See accompanying text for details.

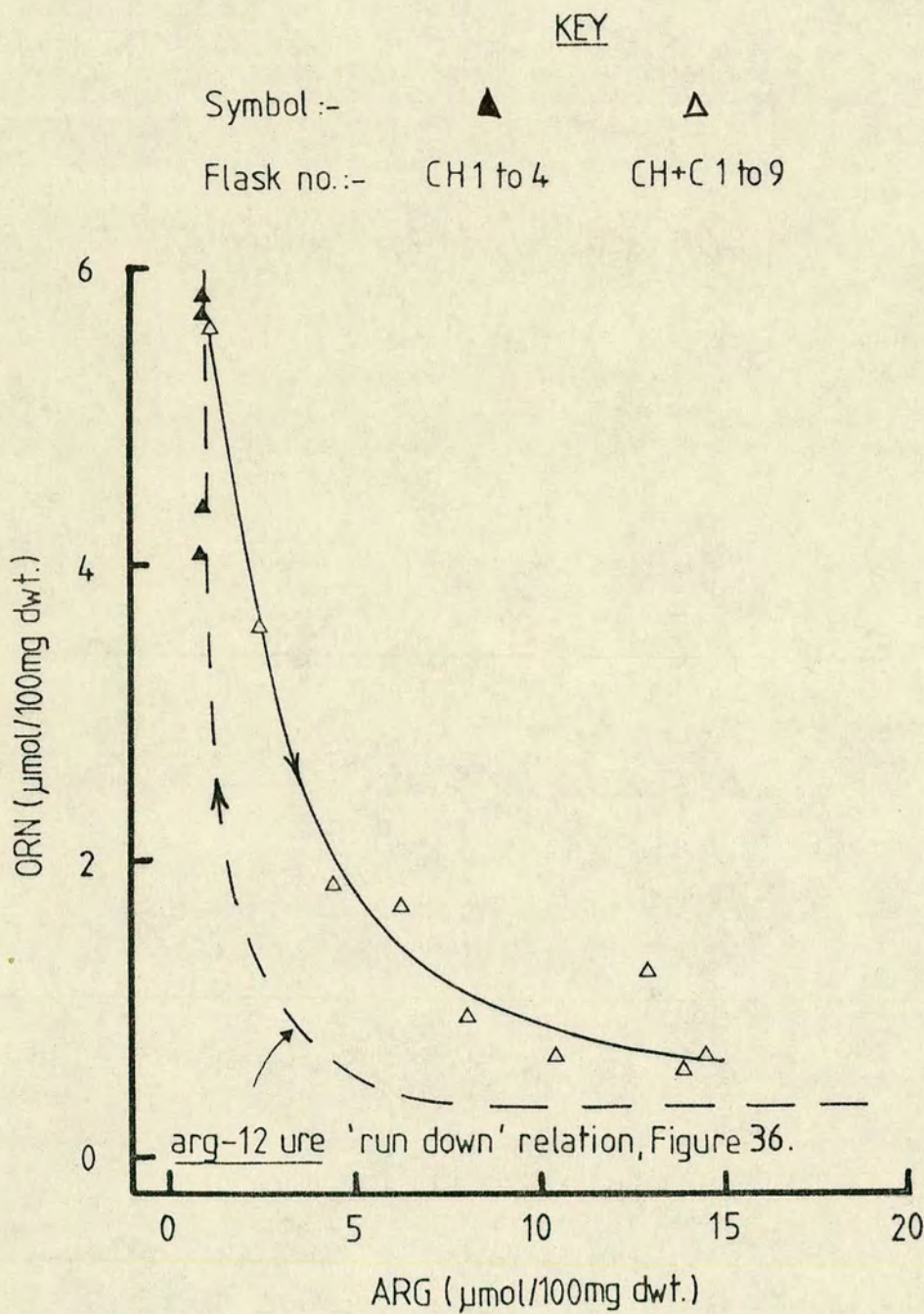


Figure 54: The "run down" relation between ARG and FxORN in arg-12 ure.

Data from Experiments Xa (○) and b(●), Tables 14 and 15, Appedix III.

See accompanying text for details.

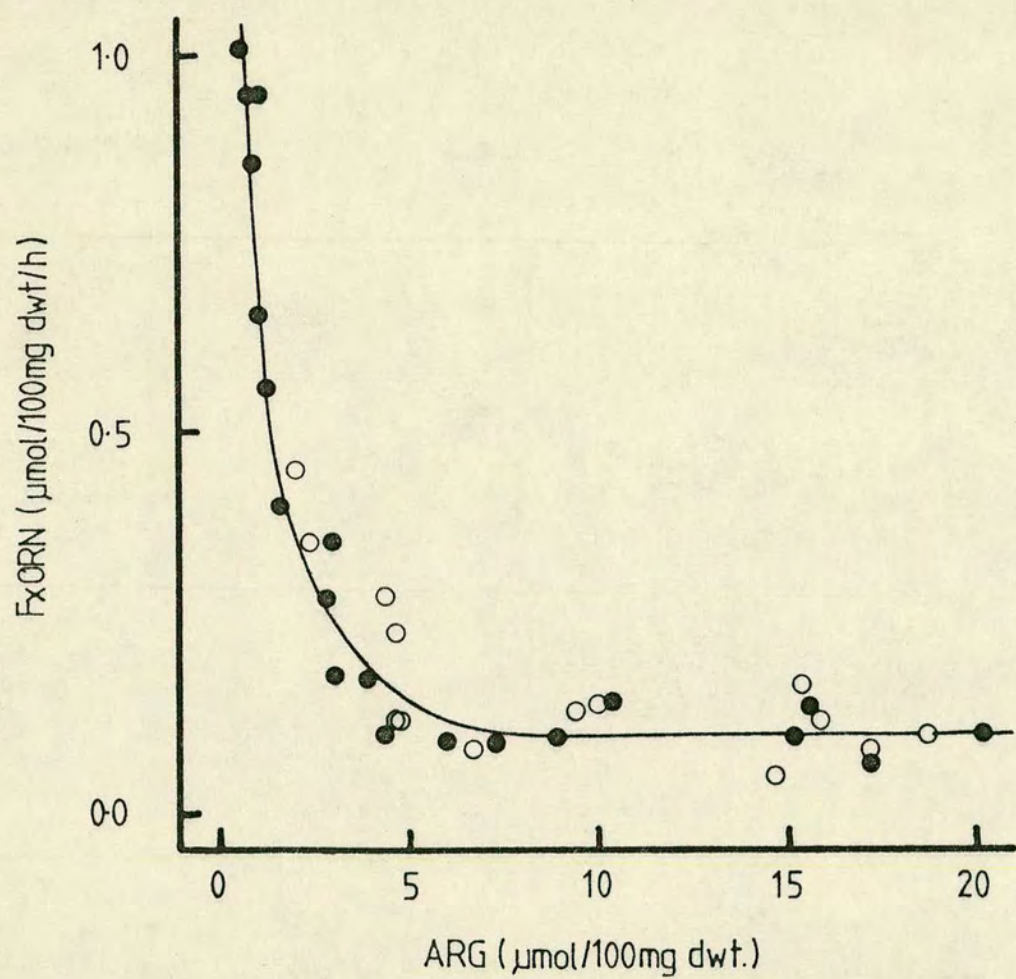
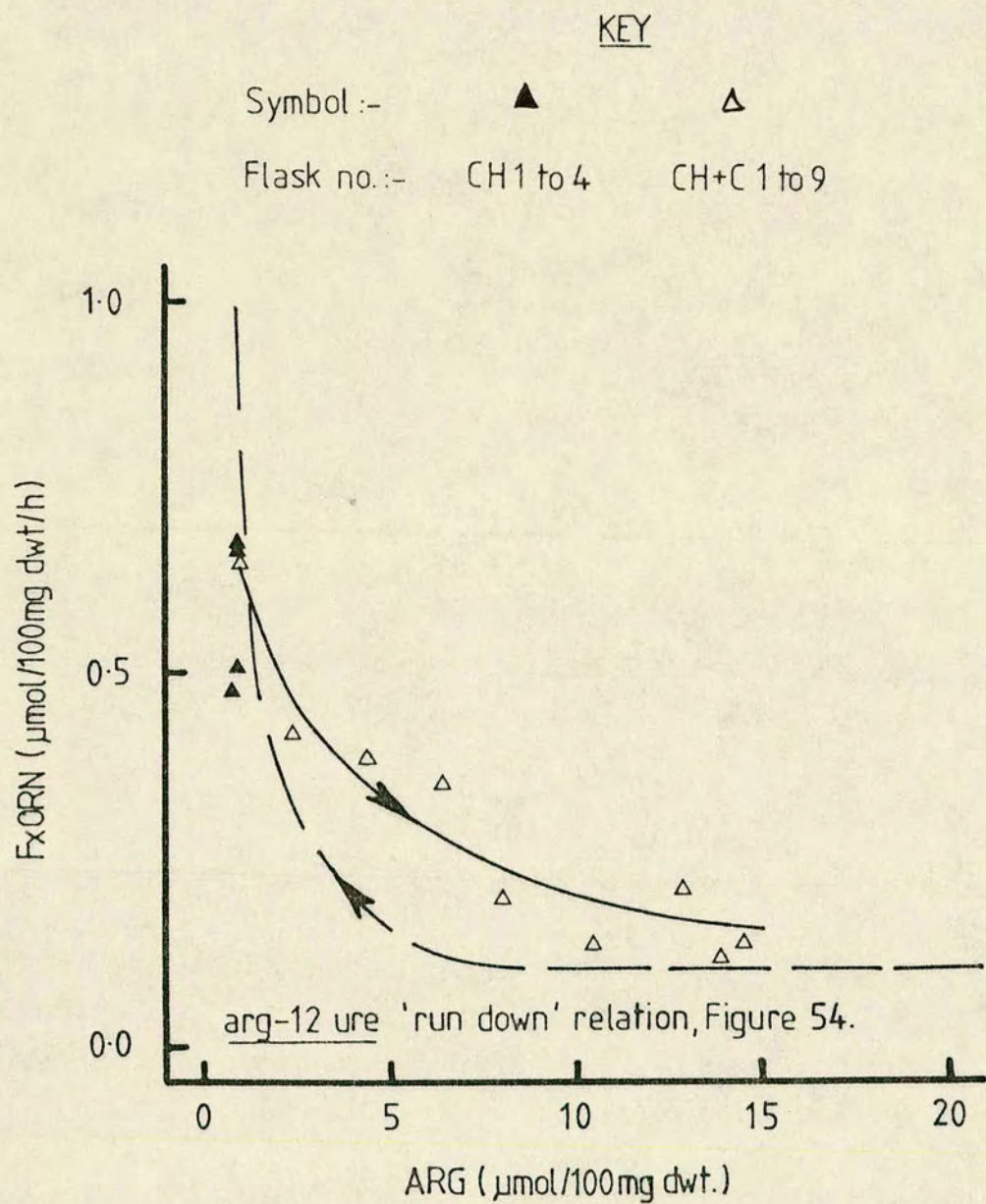


Figure 55: The "run up" relation between ARG and FxORN in arg-12 ure.

Data from Table 16, Appendix III.
See accompanying text for details.



the ARG to CIT pool relation just discussed. We have already discussed in some detail the probable and possible mechanisms underlying the ORN to ARG pool relationship described both by the "run down" and the "steady state" condition (Section 3.3). There are two intuitive reasons for expecting the "run up" condition to alter this relationship. Firstly, we expect a hysteresis in the response of ORN to the increasing level of ARG as effector since ORN accumulated at the outset of Experiment XI must be diluted out by growth and metabolism. Secondly, we inferred that the arginase flux was substantial even at low levels of total extractable ARG under the "run up" condition. In addition, the subcellular distribution of accumulated ORN will determine in part its own fate following the increase in ARG pool. The actual form of the "run up" relation should reveal information about this distribution and the relative contributions to ORN of ARG, firstly, as effector on the "proximal" part of the pathway and, secondly, as catabolic substrate.

The ORN to ARG pool plot for the data from Experiment XI is shown in Figure 53 with the "run down" relationship from Figure 36 superimposed. The figure shows that there is indeed a substantial hysteresis in the ORN pool decay under the "run up" condition. However, again the more appropriate plot for comparison is the ORN expansion flux as a function of the ARG pool. This function is shown in Figure 54 for the data from Experiments Xa and b and in Figure 55 for the data from Experiment XI with the "run down" relationship superimposed.

In fact the hysteresis observed is far less than ^{that} expected if the disparity in the ARG to CIT relations between the two conditions is to be explained by differences in the arginase flux. This is so even if it is assumed that on expansion the cytosolic ARG pool completely inhibits ORN biosynthesis (Davis et al, 1978a and b).

In addition to finding a rapid and complete cessation in ORN biosynthesis following the transfer of ure from MIN to ARG supplemented medium, Davis et al (1978a and b) also report that pre-existing ORN is very rapidly catabolised. They infer that cytosolic ARG inhibits the uptake of cytosolic ORN into the mitochondria and vesicle and may also promote the egress of ORN from the vesicle. The results from the present work are certainly compatible with this notion. ARG derived ORN has been shown to be predominantly catabolised further by OTAase (Karlin et al, 1976; Bowman and Davis 1978a and b). From a visual inspection of Table 16 there is certainly no indication in the PRO pool of such an elevated flux in Experiment XI. This may simply mean that under these conditions GSA is predominantly oxidised to GLU, as suggested also to be the case under the "run down" condition (Section 4.3.3).

In conclusion, it is clear that the ORN to ARG pool relation is a complex integration of the metabolic and effector contributions of ARG and the consequences of the compartmentation of the ORN pool on the entry and exit fluxes.

In the final analysis the actual movement of ORN pool will depend upon the contemporaneous interplay of all these factors. In the absence of detailed tracer studies or the further

metabolic dissection of the system their relative contributions cannot be judged.

In summary, it is clear from the ARG to CIT and ORN to ARG relations and the lag in adjustment of enzyme activities to changing effector levels that we are not at steady state under the "run up" condition particular to Experiment XI, but are observing a transient state and slow adjustment to a new steady state.

Comparison of the "run up" with the "run down" demonstrates the complexity of the system response to a change in metabolic influx. The change from one steady state to another is an integrated systemic response to contemporaneous and continuous changes in the levels of enzymes, their substrates and effectors and their subcellular distribution. The differences observed between the two conditions described in the responses of "controlled" components demonstrates clearly the dependence not only upon the magnitude and rate of change of each "controlling" component, but also upon the direction of that change. The discussion of the relative contributions to each response of the different factors believed to be involved has been limited by nature of the experimental system and the number and type of experimental analyses performed. However, the further dissection of the system by the introduction of mutational blocks to specific fluxes should largely obviate such problems without necessitating the resort to a more complex analytical approach. Experiments designed to this end are described in the following section.

The "run down" condition in arg-12 ota aga

In the preceding experiments with the arg-12 ure strain the interpretation of pool responses to modulation of the system influx was limited by the inability to quantify the relative contributions of anabolic and catabolic fluxes. In particular, the effect of the "distal" moiety on the "proximal" through the effector role of ARG was complicated by the unknown catabolic contribution of ARG to ORN and the dubiety attached to the estimation of the OTAase flux from the accumulation of PRO. Detailed radioisotope label equilibrium and pulse label studies of the type conducted by Flint (1977) and by Davis and colleagues (Karlin et al., 1976, and Bowman and Davies, 1977a and b) can go a long way to overcoming such difficulties. However, such an approach is by nature laborious, particularly where subcellular distributions of metabolites change with changes of metabolic state. An alternative and much simpler approach to the problem was outlined in the experimental strategy (Section 3.2), namely to dissociate completely the "proximal" and "distal" moieties by introducing the aga mutation in place of the ure mutation (Figure 7). Thus the flux to ARG reduces to the sum of the ARG pool expansion flux and the flux of ARG to protein. Now the only contribution of ARG to the "proximal" flux is the indirect effect through the operation of feedback inhibition and derepression. By also introducing the ota mutation the flux to ORN reduces to the sum of the flux to expansion of ORN pool itself and the ODCase flux (Figure 7). We can now quantify the indirect effect of ARG on the biosynthetic flux by conducting the "run down" experiments of the same type as described for the arg-12 ure. In addition, by comparison with the arg-12 ure pool data the OTAase and arginase fluxes in the

arg-12 ure strain can be estimated.

Three "run down" experiments, XII, XIII, XIV employing the arg-12 ota aga strain were conducted. The general method is by now well described and need not be repeated here. Any significant differences, however, will be mentioned. The results from the three experiments can be conveniently discussed together.

The complete experimental details for Experiments XII, XIII and XIV are given in Tables 17, 18 and 19, respectively, in Appendix III to the thesis. The only common difference in method from the arg-12 ure "run down" experiments (Tables 14 and 15) is that the arg-12 ota aga conidia were inoculated into medium supplemented with only 0.04 mM CIT (as opposed to 0.40 mM CIT). More CIT (0.36 mM) was added at the time of HIS addition. It will be shown (Section 4.3.7.1) that CIT uptake is concentration dependent at this initial level of supplementation. Consequently the mycelial CIT pool is much lower than the corresponding starting level in the arg-12 ure "run down" experiments where the rate of CIT uptake was nearly saturated. This meant that lower CIT pools (and, consequently, ARG pools) could be reached more rapidly than if 0.40 mM CIT had been added at inoculation.

In Experiment XII a 2:1 HIS:CIT was employed. In this experiment, because of the low conidial inoculum used, the mycelial contents of more than one growth flask were pooled for analysis at early time points. In Experiment XIII the HIS:CIT ratio was increased to 3:1. In Experiment XIV first a 2:1 HIS:CIT ratio was established and then, some 10 h later, the ratio was increased to 4:1. The salient features of the three experiments can be summarised briefly as follows:

(i) Growth rate

The growth rates in Experiments XII, XIII and XIV are shown in Figures 56, 57 and 58 respectively. In each experiment the growth was exponential although the rates varied between experiments in relation to the time of HIS addition and ratio of HIS:CIT. In Experiment XIV the rate fell substantially about one mycelial doubling after the increase in medium HIS:CIT ratio from 2:1 to 4:1. Growth effects of this type have been discussed in Section 4.1.6. They do not interfere with the interpretation of the experimental results. However, the differences in growth rates must be taken into account when comparing pool data from different experiments.

(ii) Mycelial aminoacid pools

The time course of changes in the HIS, CIT, ARG and ORN pools in Experiments XII, XIII and XIV are shown in Figures 59, 60 and 61 respectively. The following general points can be made concerning the variation in pools:

a) The rate of CIT uptake is not saturated at the initial level of supplementation. This conclusion follows from the low level of mycelial CIT observed in the C1,2,3 harvest from Experiment XII compared with that characteristic of arg-12 ure cultures supplemented with 0.3 mM CIT or more (Tables 7 and 9). The low mycelial CIT pool observed in harvest C1,2,3 cannot be explained by the exhaustion from the medium of exogenously supplied CIT.

This point will be developed later in the text.

b) The rate of fall of ARG relative to the rate of fall of CIT is less than in arg-12 ure. This must be due to the absence from arg-12 ota aga of the flux out of ARG via arginase. This strain difference is

Figure 56: Experiment XII: growth rate.

Data from Table 17 , Appendix III.
See accompanying text for details.

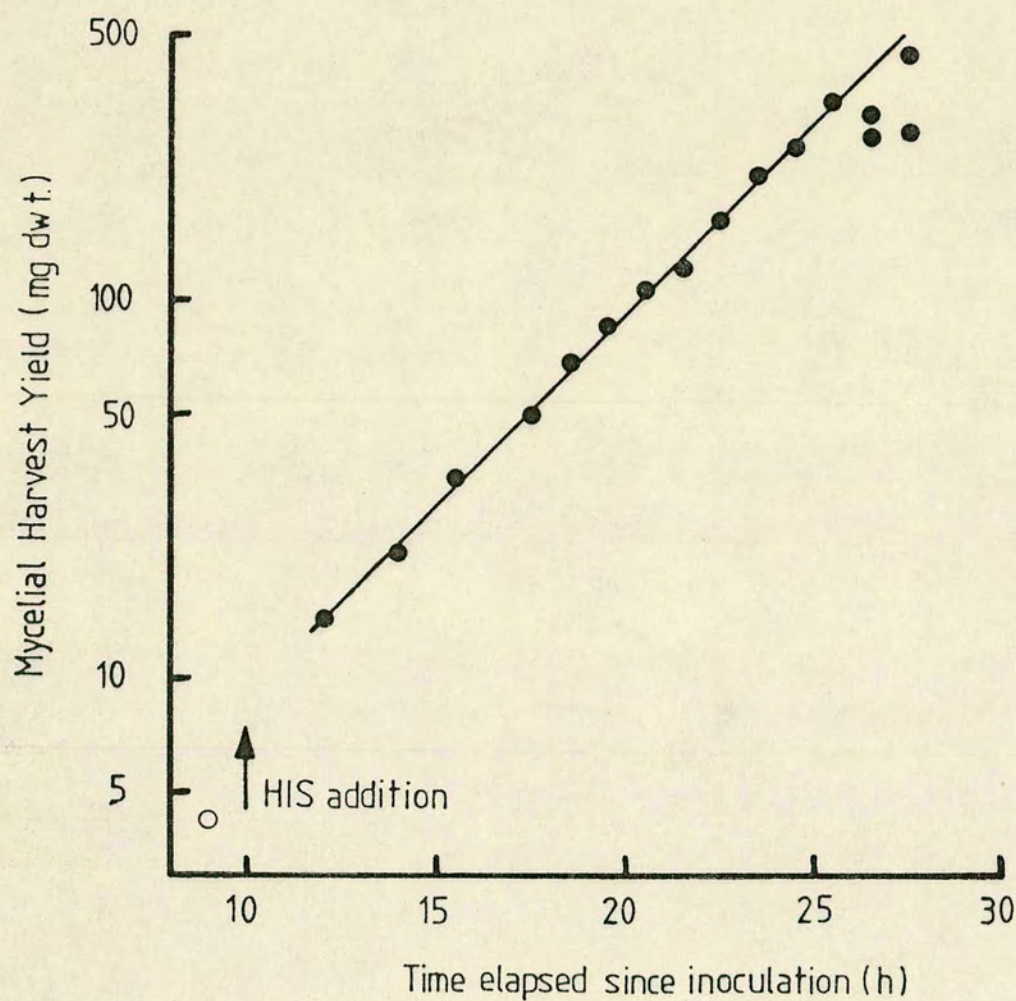


Figure 57: Experiment XIII: growth rate.

Data from Table 18, Appendix III.

See accompanying text for details.

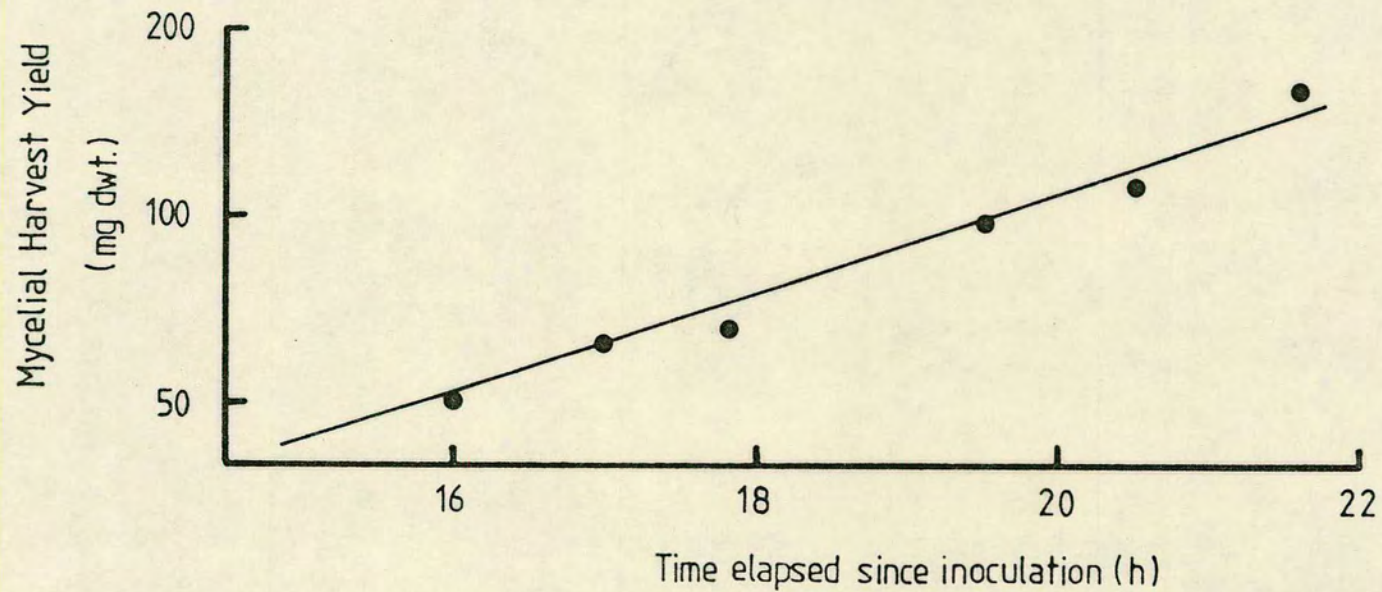


Figure 58: Experiment XIV: growth rate.

Data from Table 19, Appendix III. See accompanying text for details.

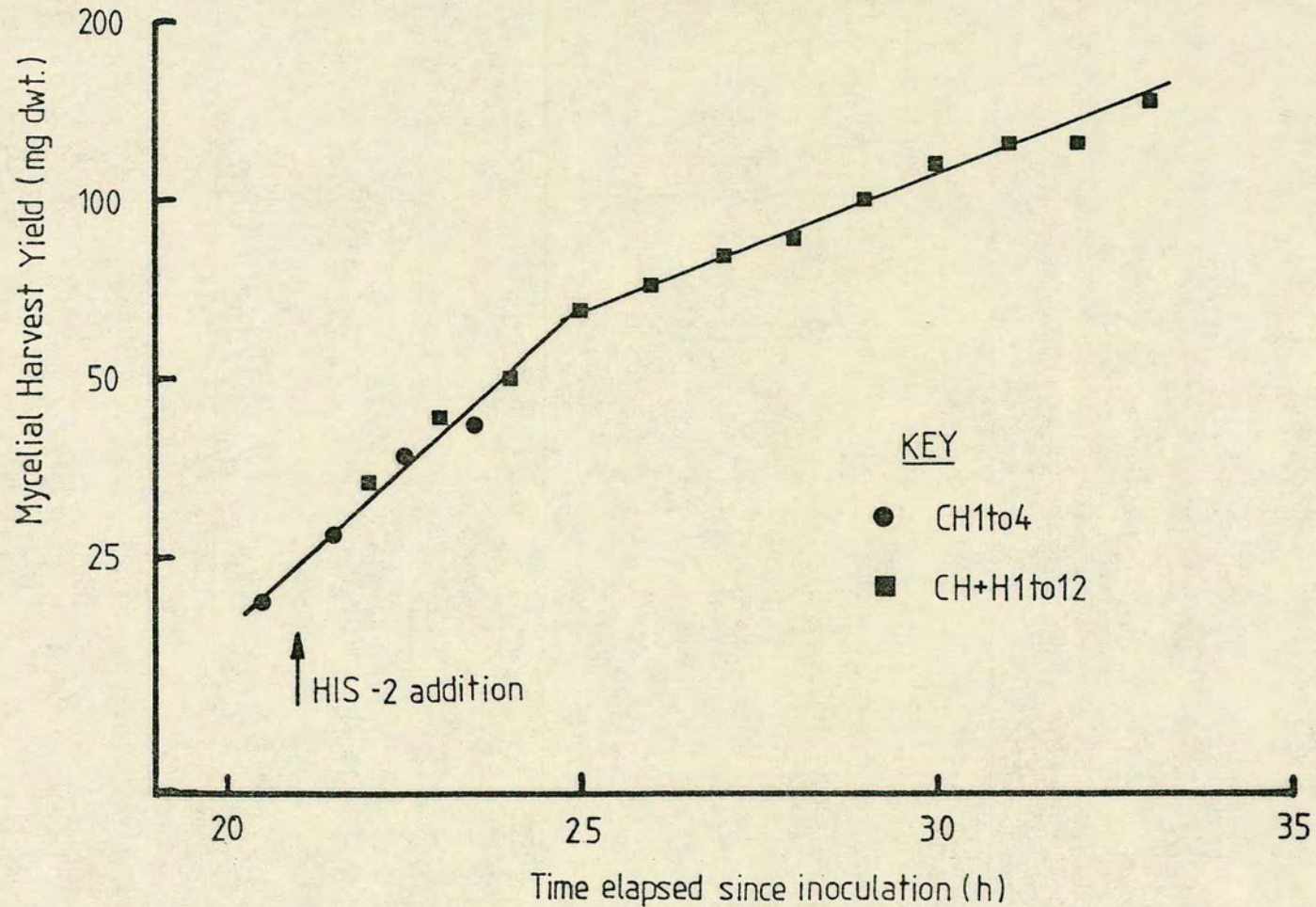


Figure 59: Experiment XII: the 2:1 HIS:CIT changes in mycelial amino acid pools.

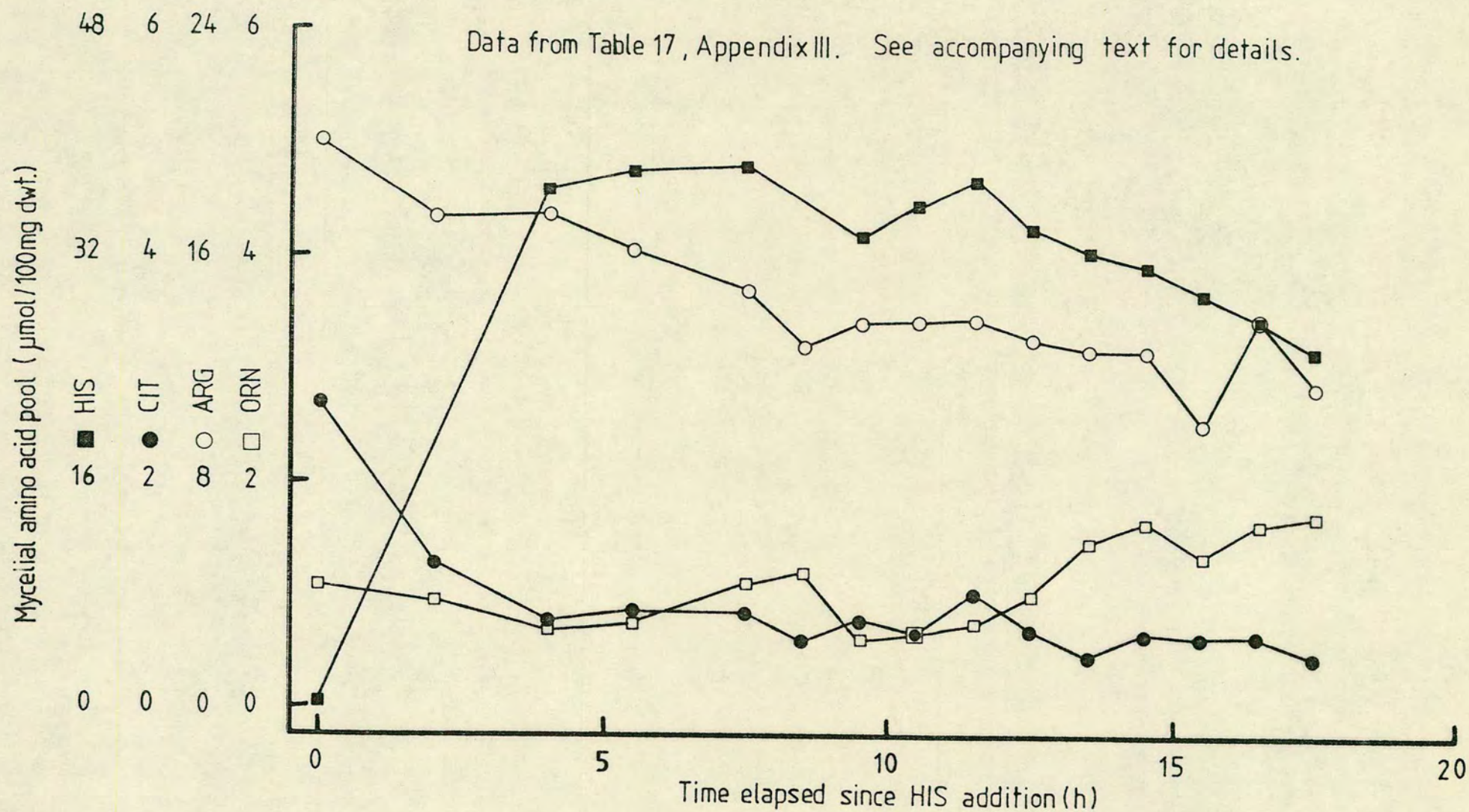


Figure 60: Experiment XIII: the 3:1 HIS:CIT changes in mycelial amino acid pools.

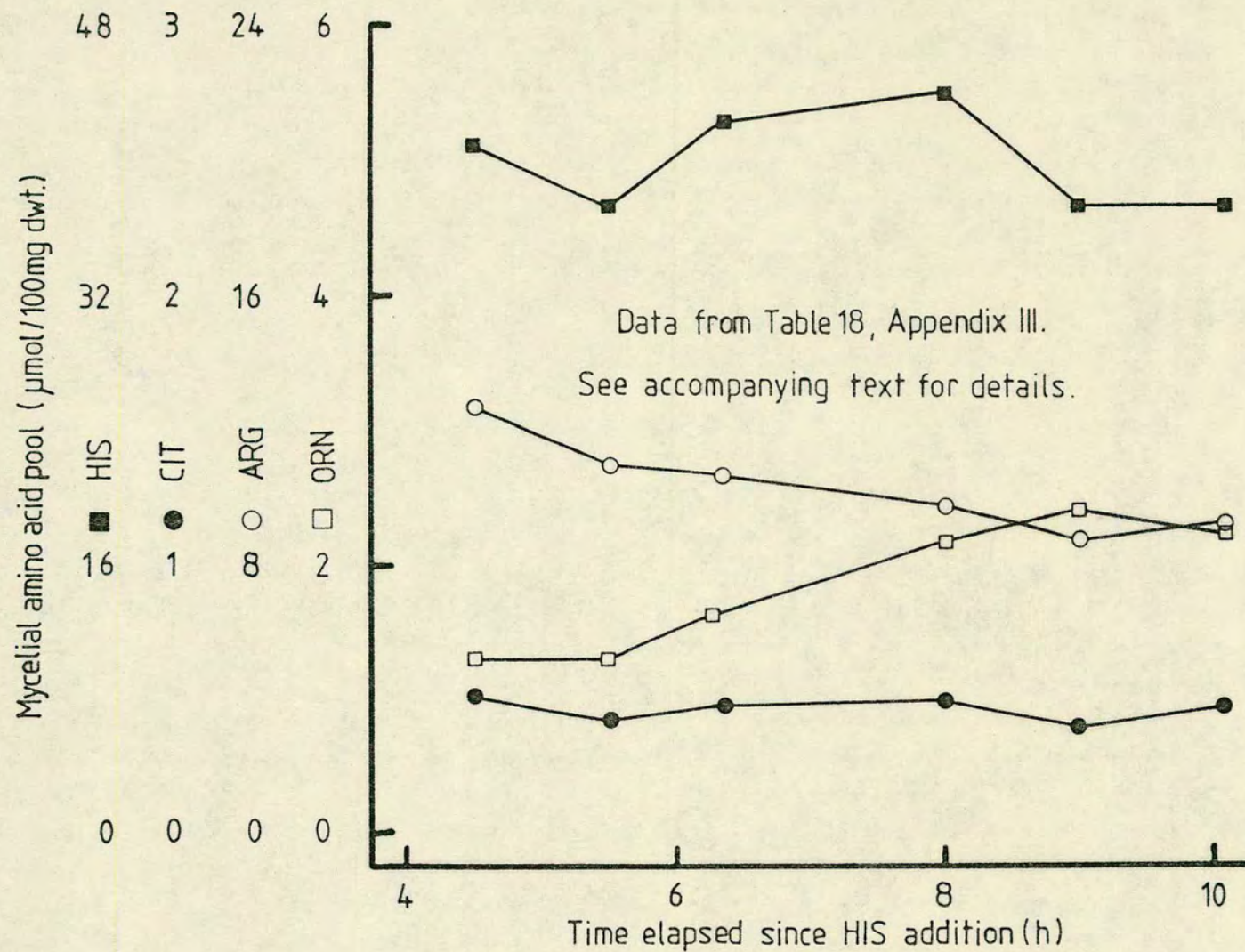
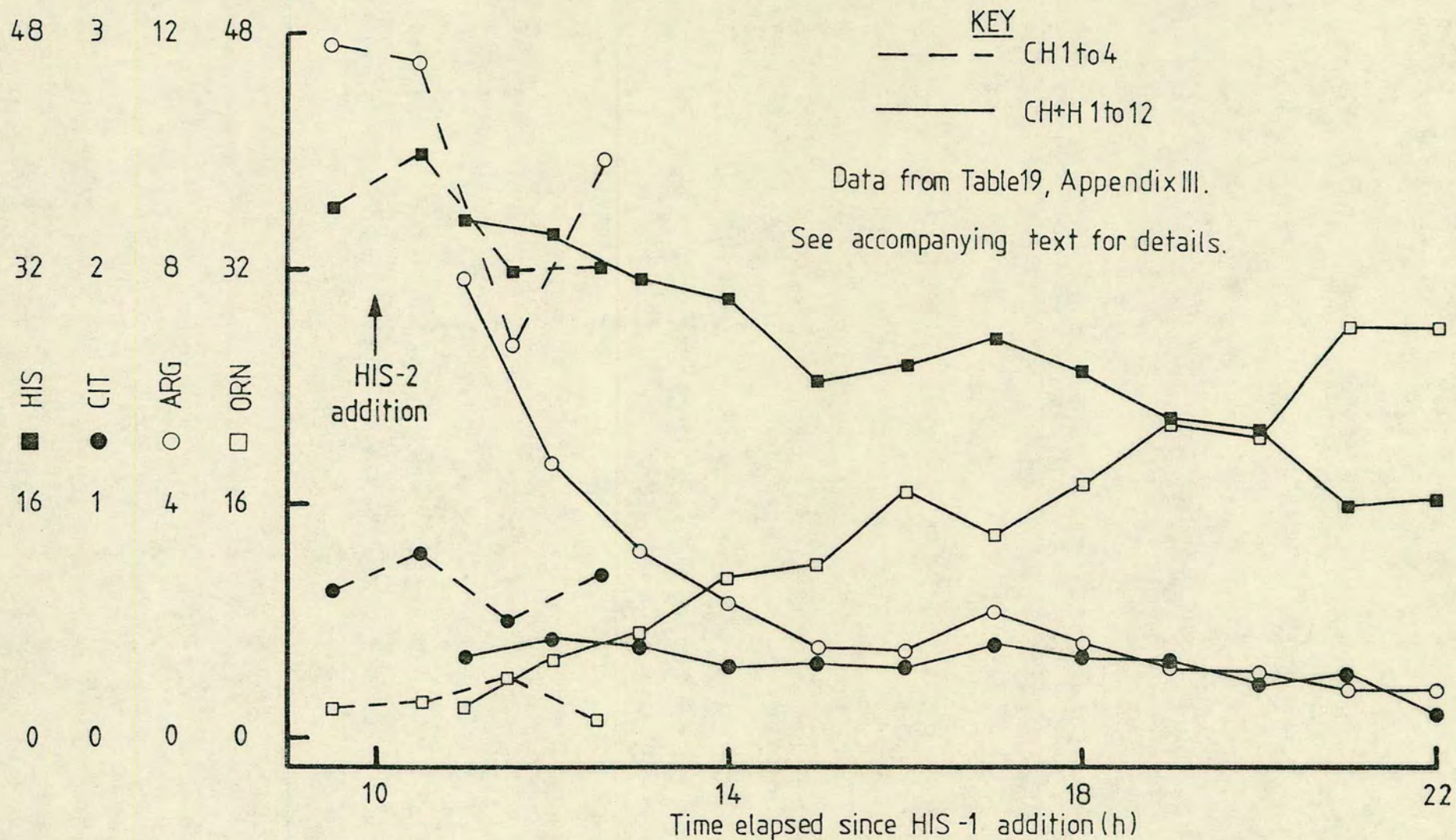


Figure 61: Experiment XIV: the 4:1 HIS:CIT changes in mycelial amino acid pools.



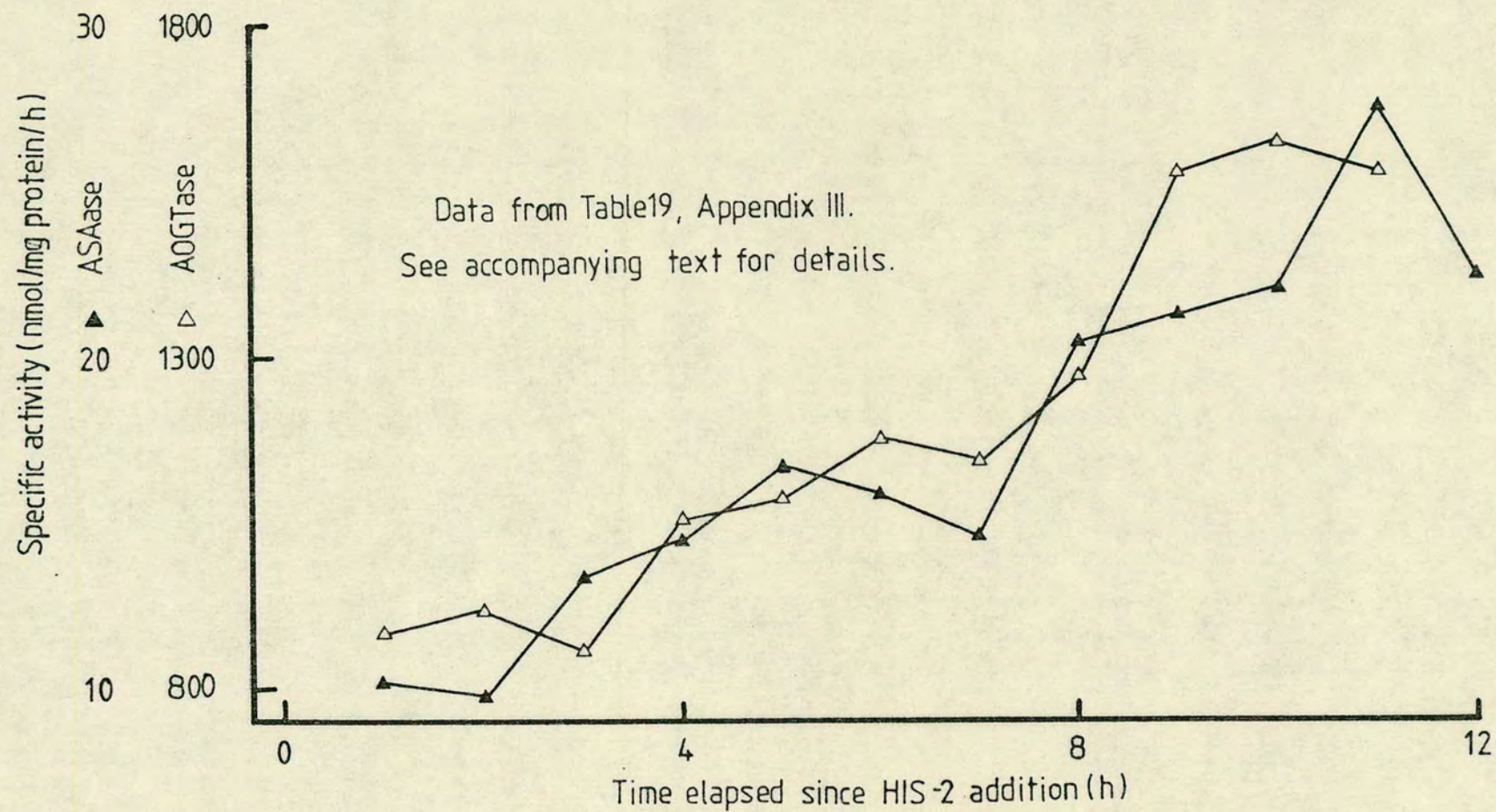
seen most clearly by comparison of Experiment XIII with arg-12 ota aga (Figure 60) with Experiment VIII using arg-12 ure (Figure 20).

Identical conditions of HIS:CIT supplementation were used in the two experiments but the ARG pool "run down" differed significantly between the two; with arg-12 ure a time invariant ARG pool of about 5 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ was reached within 6 h after the addition of HIS whereas with arg-12 ota aga the ARG pool was still falling and had only reached about 9 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ at 10 h post-HIS addition. The arginase flux in arg-12 ure can be calculated from this difference in ARG pool response. This will be shown later in the text (Section 4.3.7.3).

c) The range of ARG pools generated in each experiment overlap with the next. The overall range is essentially the same as that generated in arg-12 ure. Notably, ARG pools within the "derepression range" were reached in Experiment XIV (Table 19, Figure 61).

d) The ORN pool shows a marked and sustained increase as the ARG pool falls to below about 13 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ (Figure 61). This contrasts with the lack of ORN response in arg-12 ure until the ARG pool falls to below about 5 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ (Figure 36). Presumably, part of ^{the} indirect effect of ARG through deinhibition of the "proximal" part was masked in arg-12 ure by the direct and "opposite" metabolic contribution of ARG between 13 and 5 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ since no ORN pool response was observed in Experiments Xa or b over this range. A very dramatic and continuing increase in the ORN pool is seen in arg-12 ota aga as the ARG pool falls below 5 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ This strain difference in ORN pool response to ARG must mean that in arg-12 ure there is a varying but substantial flux through OPAase over the range of ARG and ORN pools generated.

Figure 62: Experiment XIV: the 4:1 HIS:CIT changes in enzyme specific activities.



This conclusion confirms the supposition that the accumulation of PRO in arg-12 ure only gives a qualitative indication of the OTAase flux. With a knowledge of the arginase contribution, from the difference in ARG pool response, the OTAase flux in arg-12 ure can be calculated from the difference in the ORN pool response between arg-12 ota aga and arg-12 ure. This will be demonstrated in Section 4.3.7.3. Because the metabolic connections between the "proximal" and "distal" moieties are completely severed and the OTAase flux is blocked in arg-12 ota aga, the biosynthetic flux to ORN can be quantified and, from the relation with the ARG pool, the kinetics of deINHibition can be determined. This will also be shown in Section 4.3.7.3.

e) The total lack of movement in the PRO pool despite the dramatic variation in the ORN pool confirms the absence of OTAase activity in the arg-12 ota aga strain.

(ii) Enzyme specific activities

The specific activities of AOGTase and of ASAase were determined in mycelial extracts of cultures CH + H 1 to 12 from Experiment XIV and are plotted against the time elapsed since HIS-2 addition in Figure 62. The two specific activities are modulated in a closely coordinated manner increasing approximately two-fold over the experimental time course.

Both specific activities appear to increase significantly before the ARG pool falls to within the "normal" derepression range (Figure 3). This apparent "shift" in the derepression curve will be discussed in Section 4.3.7.2.

Figure 63: Experiment XII: the 2:1 HIS:CIT rate of medium CIT and HIS depletion.

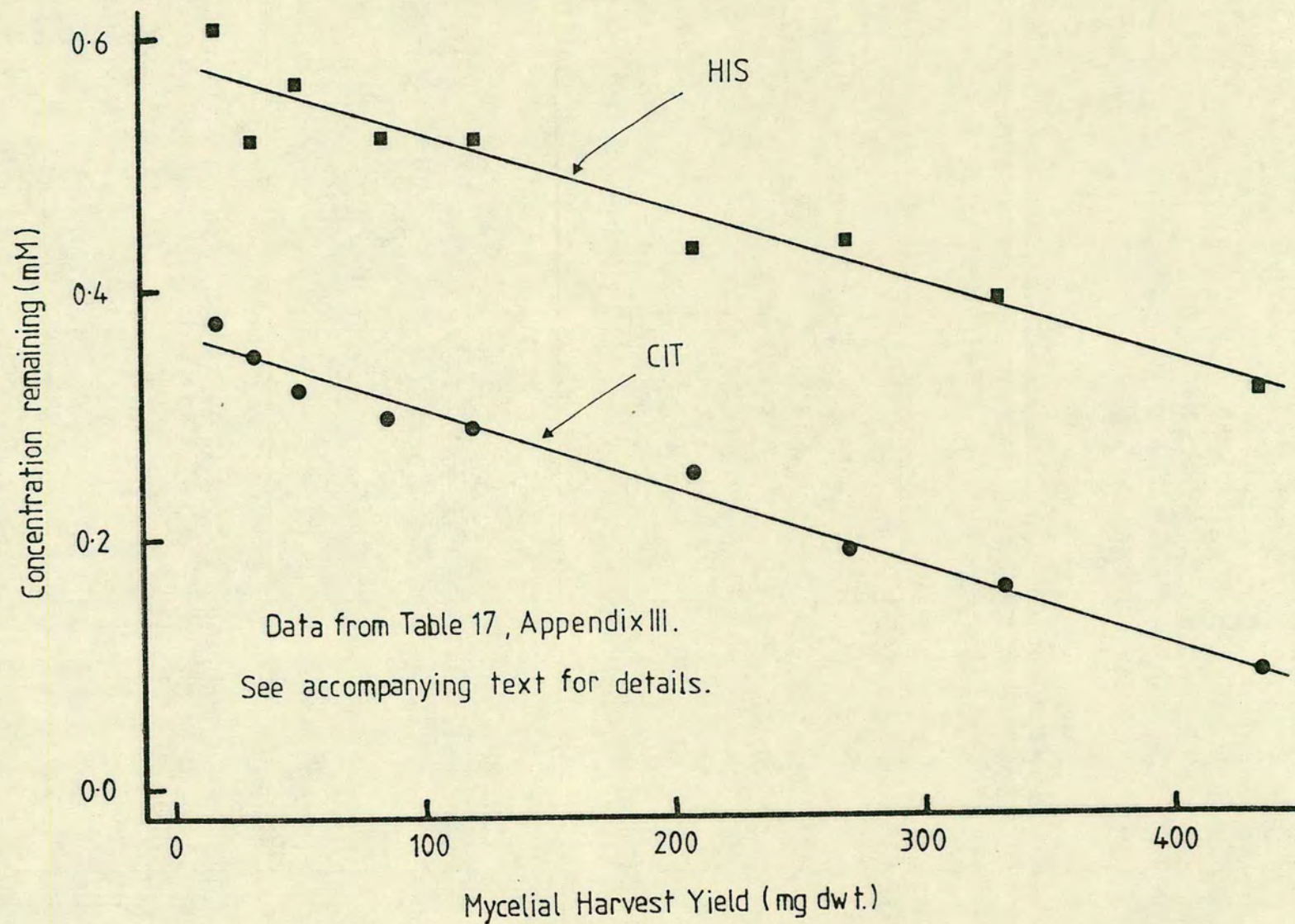
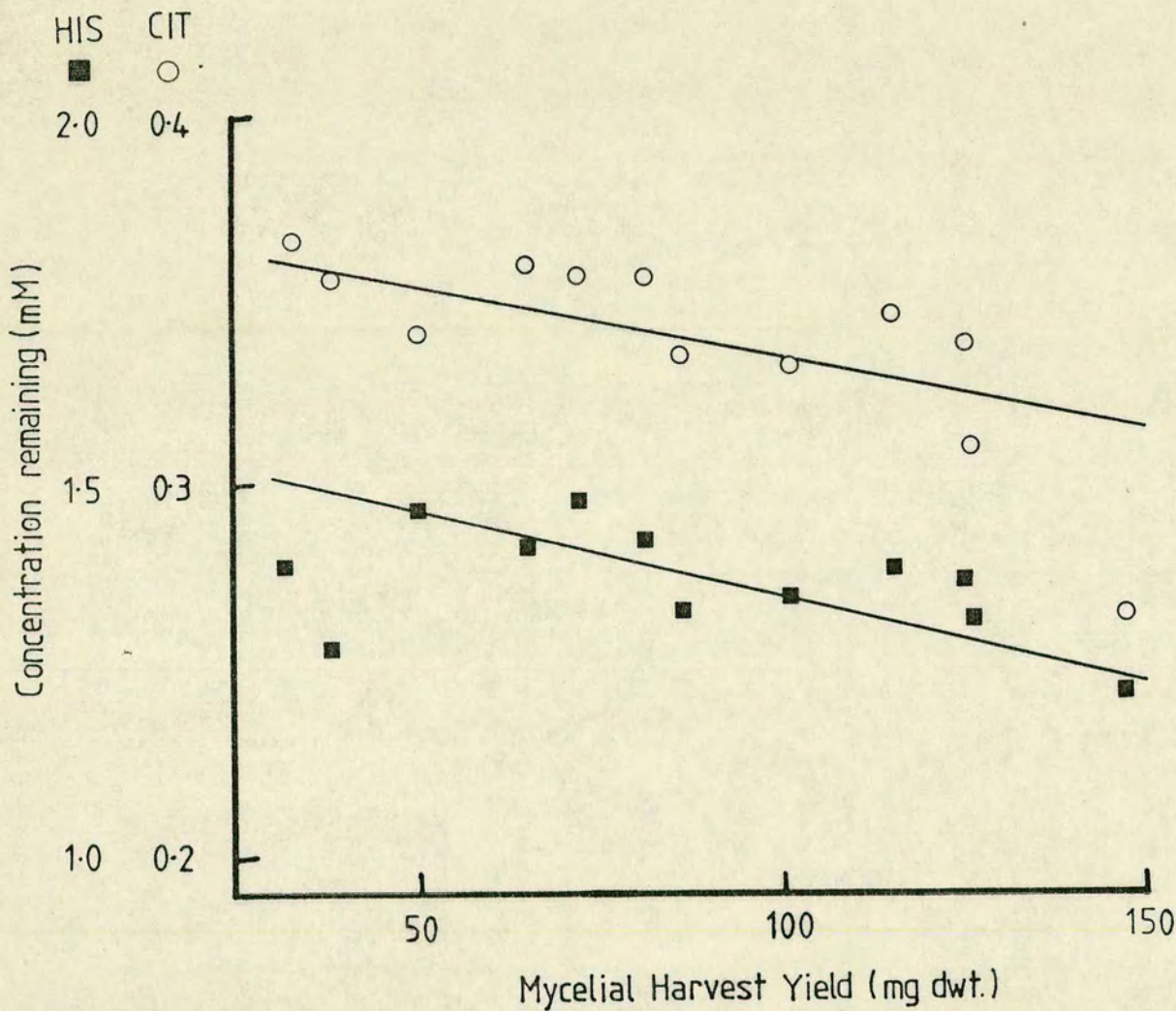


Figure 64: Experiment XIV: the 4:1 HIS:CIT rate of medium
CIT and HIS depletion.

Data from Table19, Appendix III.

See accompanying text for details.



(iii) Medium depletion rates

The concentration of HIS and of CIT present in medium samples taken immediately before the harvest of cultures from Experiments XII and X were determined by the automated colorimetric methods described in Sections 2.2.8.1 and 2.2.8.2. Figure 63 shows that both HIS and CIT were depleted at constant rates over the entire time course of Experiment XII and, moreover, that under the supplementation regime particular to that experiment, HIS and CIT were depleted at about the same rate of $25 \mu\text{mol. per } 100 \text{ mg. d.wt. increase, equivalent to } 5.78 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. h}^{-1}$. With this value for the metabolic influx we can calculate at what point the new metabolic steady state has been reached by summation of the fluxes beyond CIT uptake. This calculation will be described along with others of the same nature in Section 4.3.7.1.

CIT is only taken up by the "general" amino acid transport system (System II) in Neurospora (Thwaites and Pendyala, 1969). The equal rates of depletion at a 2:1 ratio of HIS:CIT demonstrates that the affinity of CIT for this uptake system is higher than the affinity of HIS. The difference in affinities must be more than 2-fold since HIS is also taken up by systems other than the "general" (Pall, 1970).

The relative rates of HIS and CIT depletion by arg-12 ota aga at a 4:1 ratio of medium HIS:CIT are shown in Figure 64. The data come from cultures CH + H to 1 to 12 from Experiment XIV. In contrast to the same analysis in Experiment XII (Figure 63) these data do not describe smooth, linear depletions. This is believed to be due largely to experimental error; considerable difficulties were encountered in the mechanics of these determinations. The

rates are estimated to be 90 and 16 $\mu\text{mols. per } 100 \text{ mg d.wt. increase}$ for HIS and CIT respectively.

In summary, the range of ARG pools generated in these three "run down" experiments with arg-12 ota aga closely matches the range established in Experiments Xa and b with arg-12 ure . The differences in relative ARG and ORN pool movements between arg-12 ure and arg-12 ota aga indicate the arginase and OTAase fluxes in the former strain. The determination of these fluxes will be shown in the following and final sub-section to the Results and Discussion. The effector role of ARG is clearly shown in the dramatic increase in the ORN pool as the ARG pool falls to below $13 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ By removing the proportion of this flux increase which is attributable to derepression the kinetics of deINHibition can be determined directly from the ARG and ORN pool data. This will also be shown in the following comparison of the arg-12 ure and arg-12 ota aga data.

3.7

A comparison of the arg-12 ota aga and arg-12 ure systems; the analysis of the control of flux.

The contents of this sub-section, which concludes the "Results and Discussion" divides conveniently into three principal topics. Firstly, we will show that, like the arg-12 ure system, the arg-12 ota aga "rundown" condition presents a quasi-steady state and we will also see what conclusions with regard to the steady stateness of our system and to "unknown" fluxes

may be drawn from a "balance sheet" analysis of both the arg-12 ota aga and the arg-12 ure data. Secondly, we will continue the discussion, started in Section 4.3.6 of the derepression of AOGTase and ASAase in arg-12 ota aga. Finally we will discuss the pool to pool and pool to enzyme correlations generated by the arg-12 ota aga data and compare them with those already established with the arg-12 ure system. From such comparisons we shall be able to calculate several of the fluxes through the ARG pathway. In addition, we will be able to interpret, more clearly than permitted by consideration ^{of} the arg-12 ure data alone, the factors of importance in the control of those fluxes.

4.3.7.1

The quasi-steady state of the arg-12 ota aga "rundown" condition and the "balance sheet" analysis.

- a) The quasi-steady state of the arg-12 ota aga "rundown" condition.

Before proceeding with a comparative analysis of the arg-12 ota aga and arg-12 ure data we must establish that the arg-12 ota aga system is at a quasi-steady state under the "rundown" condition. We established this to be essentially true for the arg-12 ure "rundown" condition by showing that the ARG to CIT and OBN to ARG relations generated were essentially the same as those described by the "steady state" condition (Section 4.3.3.1). We cannot use this method with arg-12 ota aga since we have no "steady state" data for this strain with which to compare the "run down" behaviour. However, a quasi-steady state can be shown to prevail throughout the post-HIS addition pool adjustments by an alternative analysis, as follows:

Figure 65: Experiment XII: the 2:1 HIS:CIT rate of increase in total mycelial CIT and ARG.

Data from Table 17 , Appendix III.
See accompanying text for details.

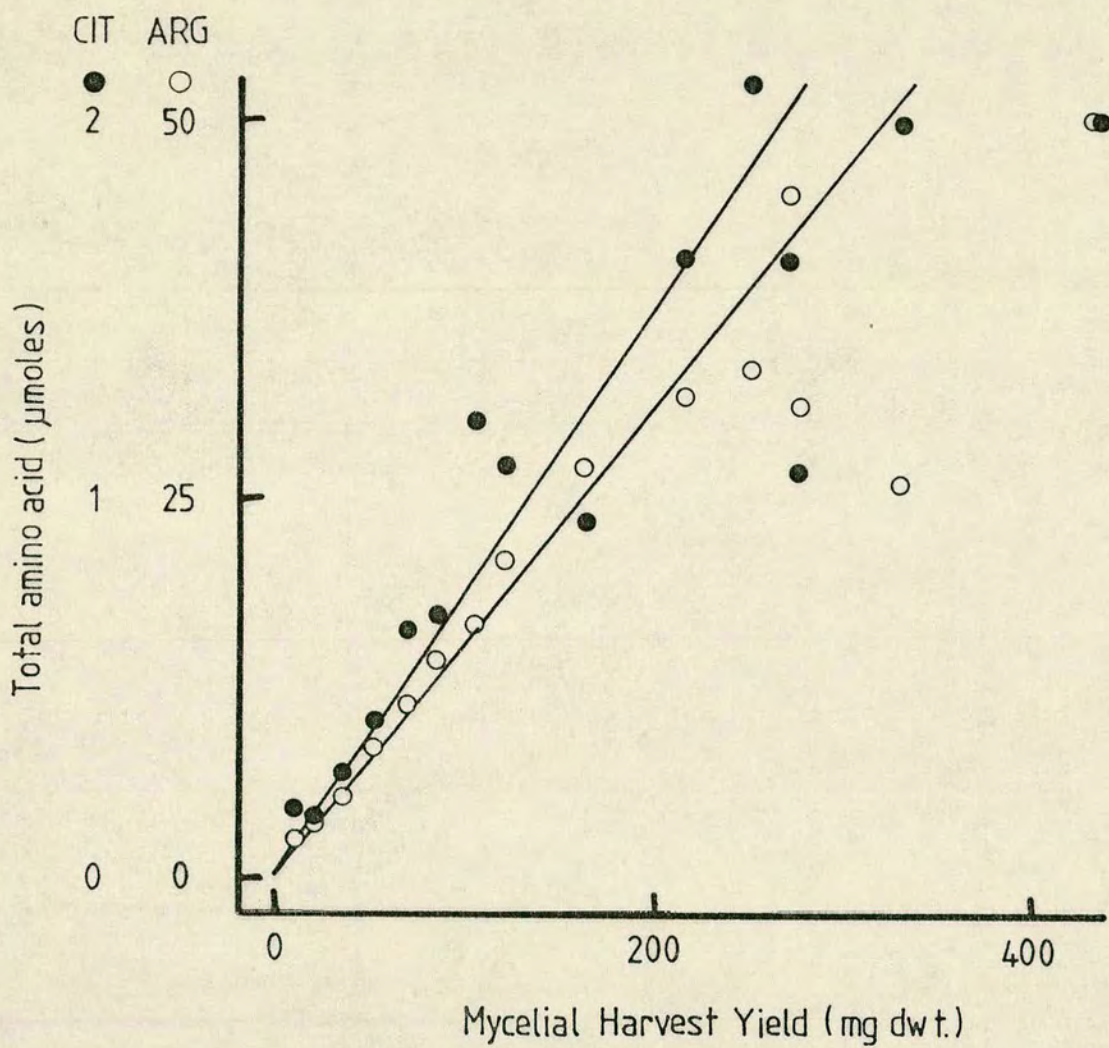
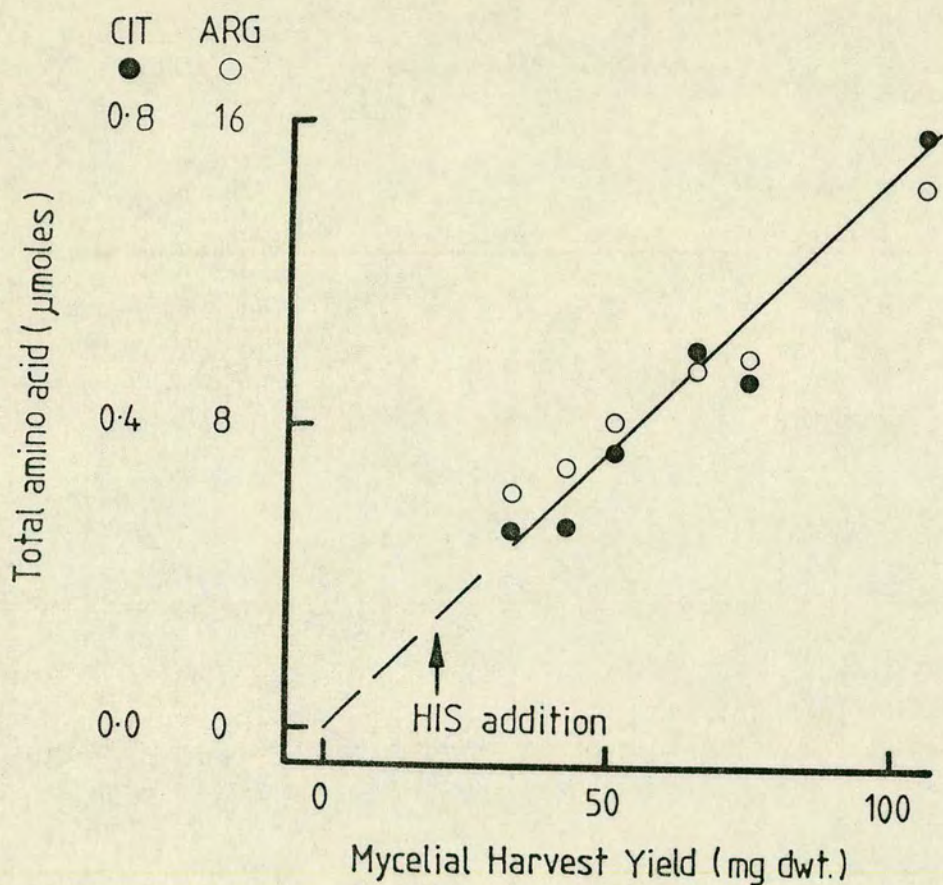


Figure 66: Experiment XIII: the 3:1 HIS:CIT rate of increase in total mycelial CIT and ARG.

Data from Table 18, Appendix III.
See accompanying text for details.



In an exponentially growing system at steady state the total amount of any metabolic will be directly proportional to the weight, with the slope through the zero coordinates being equal to the steady state concentration of that metabolite. Following a change in metabolic influx there will be a transient adjustment (during which time the system is not at steady state) before a new lower (or higher) rate of increase, i.e. steady state concentration, is reached. Therefore, by plotting the total amount of CIT and ARG (the product of the mycelial pool and yield) against the harvest yield for the data from Experiments XII, XIII and XIV we can estimate to what extent a steady state is approached in the arg-12 ota aga "run down" condition.

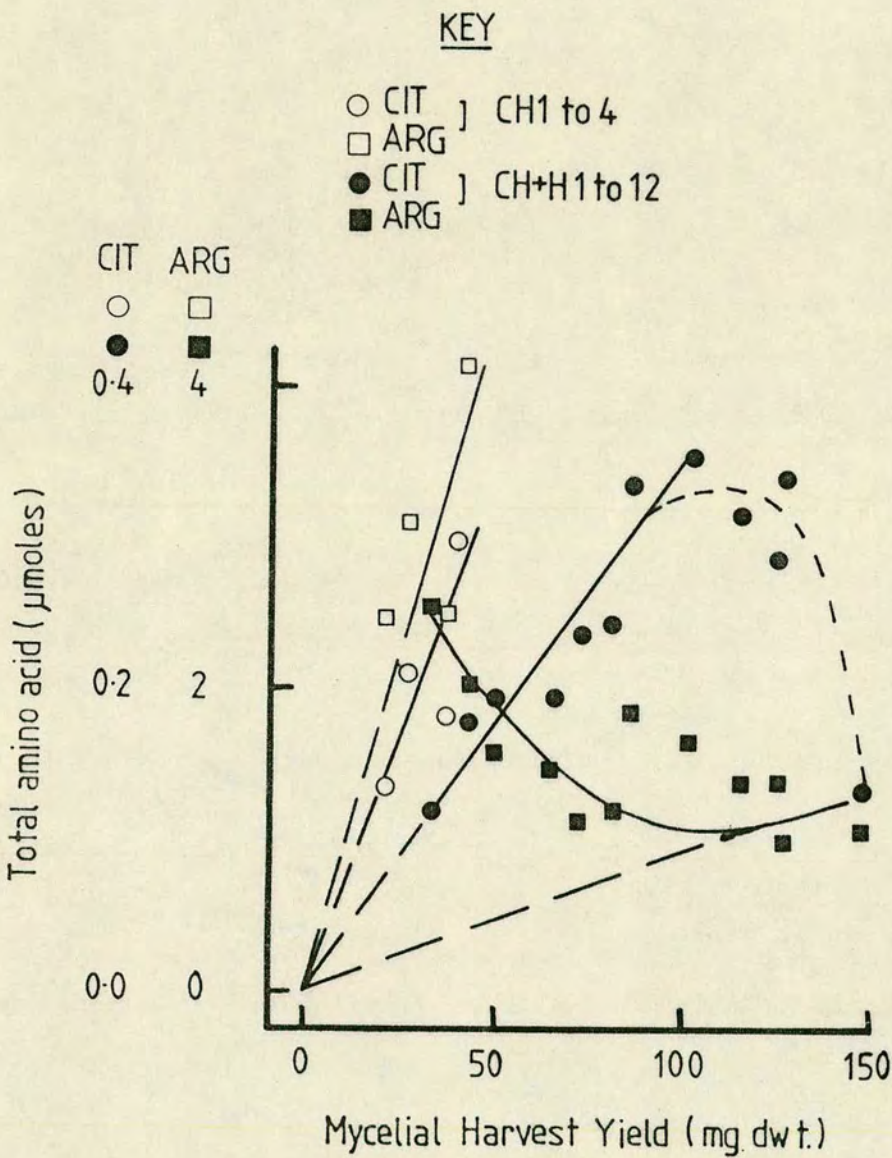
Figure 65 shows the total amounts of CIT and ARG in cultures CH 1,t2 to CH 19 from Experiment XII plotted against the harvest yields. Although there is some variation about the slopes, the slopes (i.e. concentration) for both CIT and ARG are essentially constant. We may, therefore, conclude that in this experiment the system was at a quasi-steady state throughout the post-HIS addition pool adjustments. We will show by the "Balance sheet" analysis (Part (b) to this sub-section, Example 3) that the pool regime reached at the end of this experiment must approximate closely to a final steady state.

Figure 66 shows the same plot for the data from Experiment XIII. Again, the rates of increase in total amounts of CIT and ARG are essentially constant. Again, we may conclude that the system was at a quasi-steady state throughout the post HIS addition period analysed.

Figure 67: Experiment XIV: the 4:1 HIS:CIT rate of increase in total mycelial CIT and ARG.

Data from Table19, Appendix III.

See accompanying text for details.



Finally, the same plot for the data from Experiment XIV is shown in Figure 67. Here the picture is more complex. The data for the four cultures, CH1 to 4, show the rate of increase in amount of CIT and ARG per unit weight under the 2:1 HIS:CIT condition. Although the data is limited, these cultures appear to be at a quasi-steady state. This is expected since, under the same conditions of HIS:CIT supplementation, a quasi-steady state was clearly established in Experiment XII (Figure 65).

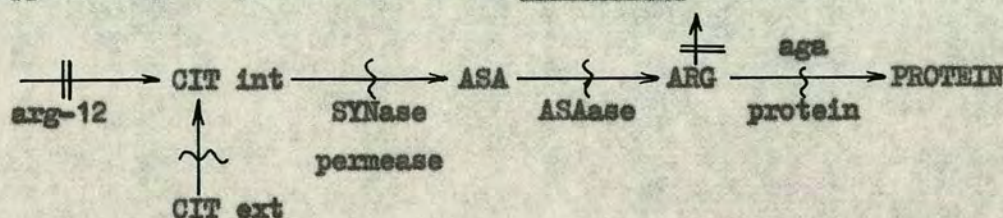
In those cultures where the HIS:CIT ratio was increased to 4:1 (CH + H1 to 12) the rate of increase in the amount of CIT fell almost immediately to a significantly lower rate. The data suggest that the rate may fall again at a later point in the experimental time course. This apparent second fall coincides approximately with the derepression of ASAase (and AOGTase) (Figure 62) and therefore probably reflects the resultant increase in flux out of CIT rather than a fall in rate of uptake. The crash at the last point may not be "real" but "experimental", due to a lower rate of CIT uptake compared with the other cultures, since the corresponding concentration of CIT remaining in the medium was unexpectedly low (Figure 64).

The transition of ARG to a new steady state following the addition of more HIS takes place slowly, with a new rate of increase in amount per unit weight, probably established towards the end of the experimental time course. The CIT and ARG pool data from Experiment XIII, which has been shown above to be at steady state, overlap with the upper range of values in the transitional phase in Experiment XIV. We will see that the ARG to CIT relation for both sets of data are essentially the same (Figure 70). We can

infer that despite the departure from true steady state behaviour over this range of CIT and ARG concentrations in Experiment XIV the system must nevertheless approach a quasi-steady state. We can also infer from a "balance sheet" analysis (part (b) to this sub-section, Example 4) that a quasi-steady state also prevails over the lower end of the ARG pool range in Experiment XIV.

b) The "balance sheet" analysis

By definition when the rate of formation of each metabolite is balanced by its rate of consumption the system is at steady state. In an exponentially growing system the flux to expansion (Fx) of the intermediate pool must be added to the transformational flux to the next metabolite in the pathway to obtain the consumption flux. Therefore in our system at steady state the concentration of exogenously supplied CIT falls and the amount of protein ARG and, in the case of arg-12 ure, urea increases with time, while the concentration of all of the intermediates remain at ^acharacteristic, time invariant, concentration. In fact we have seen that a quasi-steady state is approached even when the levels of intermediates are not time invariant, but where the relaxation times of the connecting steps are faster than the rate of change in metabolic influx. We can determine whether or not a system is at steady state by the "balance sheet" analysis. Consider the sequence of transformations from exogenously supplied CIT to protein-ARG in an arg-12 aga strain:



At steady state we have: Flux in = Flux out

Therefore:

Rate of CIT ext uptake = Rate of CIT int consumption

$$= F \times \text{CIT int} + F \text{ SYNase}$$

$$= F \times \text{CIT int} + F \times \text{ASA} + F \text{ ASAase}$$

$$= F \times \text{CIT int} + F \times \text{ASA} + F \times \text{ARG} + F \text{ protein}$$

Therefore if the sum of the intermediate pool expansion fluxes plus the flux to protein ARG balance the rate of medium CIT depletion then the system is by definition at metabolic steady state. The flux to protein ARG can be considered a constant. Since the flux to expansion of each intermediate is equal to the product of the growth rate constant, k , and its mycelial concentration it follows that all fluxes are potentially calculable if the growth rate and metabolic pools are known. Furthermore, if by another criterion a system can be judged to be at metabolic steady state then the value of an unknown flux can be calculated from the balance remaining after summing all the other component fluxes. This type of analysis is intrinsic to the calculation of fluxes from a comparison of the arg-12 ota aga with the arg-12 ure pool data, to be described in Section 4.3.7.3. Four examples of the method as applied to particular experimental data and specific questions about the system will be given here.

Example 1

Taking the data from Experiment VIIa (Table 9) we can calculate the arginase flux in arg-12 ure at saturating CIT supplementation, as follows:

We established that the two pairs of cultures supplemented with 0.5 mM and 0.3 mM CIT alone, respectively, grew at the same rate ($k = 0.289 \text{ h}^{-1}$), depleted CIT from the medium at the same time invariant, rate ($56 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. increase}$) and, consequently, were at the same metabolic steady state. Knowing what the system consumption flux must have been we can calculate the arginase flux as the balance remaining after the summation of the mean CIT and ARG pools observed, plus the estimated ASA pool and flux of ARG to protein (We have a reliable estimate of the Flux to ARG protein under these conditions from the determinations of Tateson (1971) and Flint (1977). We do not have values for the ASA pools in the cultures concerned but for the purposes of the "balance sheet" analysis this pool can be reliably estimated from the extensive data of Barthelmess et al (1974)). Therefore, we have:

$$\text{Flux in} = \text{Flux out}$$

$$\text{i.e. Rate of CIT depletion} = F \times \text{CIT} + F \times \text{ASA} + F \times \text{ARG} + F \times \text{protein ARG} + F \text{ arginase}$$

$$\begin{aligned} \therefore F \text{ arginase} &= k. (56 - (16.0 + 1.4 + 17.1 + 13.0)) \\ &= k. 8.5 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. h}^{-1} \\ &= 2.46 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. h}^{-1} \end{aligned}$$

This value is only about 2/3 of that calculated directly by Flint (1977) from urea accumulations. The reason for this discrepancy is not known.

Example 2

In Experiment XII (Table 17) with the arg-12 ota aga strain we observed a CIT pool of only $2.68 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ in the pooled cultures C1, 2, 3 from which we concluded that the rate of CIT

uptake was unsaturated at the low level of supplementation prevailing. Taking again the values for the ASA pool and the flux of ARG to protein used in Example 1 we can calculate the actual rate as follows:

$$\begin{aligned}
 \text{Flux in} &= \text{Flux out} \\
 \text{Rate of CIT uptake} &= F \times \text{CIT} + F \times \text{ASA} + F \times \text{ARG} + F \text{ protein ARG} \\
 &= (2.68 + 1.4 + 20.1 + 13.0) \mu\text{mol } 100 \text{ mg}^{-1} \text{ d.wt.} \\
 &\quad \text{increase} \\
 &= 37.18 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. increase}
 \end{aligned}$$

As concluded from the mycelial CIT pool this calculated rate is significantly lower than the saturated rate observed in Experiment VIIb (see Example 1 above). This calculated rate is equivalent to the consumption between inoculation and harvesting of 1.6 μ moles of CIT per 400 ml culture, only one-tenth of that added. This proves that the CIT pool observed is a reflection of an unsaturated rate of CIT uptake rather than the exhaustion of CIT from the medium. (Even if a saturated rate of CIT uptake and a maximally expanded CIT pool of 20 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ were invoked prior to the harvesting of these cultures this could only account for the consumption of an additional 0.75 $\mu\text{moles of CIT}..$

Example 3

Following an experimentally imposed change in the rate of CIT uptake the component fluxes comprising the rate of CIT consumption adjust to balance the new rate and re-establish the steady state equality between "Flux in" and "Flux out". The response is systemic because the steady state is a systemic property. The levels of pools will continue to change until the rate of formation of each pool is balanced by its rate of removal and the sum of the transformational and expansion fluxes equals the new flux into the system.

The time taken for the new final steady state to be attained will depend upon the time taken to dilute and consume pre-existing pools through growth and metabolism.

In Experiment XII the rate of CIT depletion fell from an estimated $37.18 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. increase}$ (Example 2 above) to a measured $25 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. increase}$ (Figure 63) following the addition of HIS and more CIT to the medium. As expected, the mycelial CIT and ARG pools fell with time subsequent to the imposition of this new, lower rate, of CIT uptake (Figure 59). We have shown in part (a) above (Figure 65) that the system is at a quasi-steady state throughout the post-HIS addition pool adjustments. We can conclude, using the balance sheet approach, that the aminoacid pool regime established by the end of the experimental time course must represent the new final steady state in response to the change in rate of CIT uptake. The proof is as follows:

$$\begin{aligned} \text{Flux out} &= F \times \text{CIT} + F \times \text{ASA} + F \times \text{ARG} + F \text{ protein ARG} \\ &= (0.45 + 1.4 + 11.4 + 13) \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. increase} \\ &= 26.25 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. increase} \\ &\approx \text{Rate of CIT depletion } (25 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. increase}) \end{aligned}$$

Therefore Flux in \approx Flux out

In the above summation the values used for CIT and ARG pool are the average for the last two simultaneously harvested cultures. The estimate of the flux of ARG to protein is the same as used before. In this case, however, the ASA pool is certainly overestimated. This pool is more likely to be of the order of $0.4 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ given the surrounding CIT and ARG pools (Barthelme et al.

1974). Thus the agreement between the measured "Flux in" and the estimated "Flux out" is very close.

Example 4

By the same calculation shown in Example 3 above, we can demonstrate that the pool regime established at the end of arg-12 ure "run down" experiment, X_b , represents the final steady state. From the concentration of CIT remaining in the medium at each harvest we estimated the post-HIS addition rate of CIT depletion to be constant at about 15 μmol per 100 mg d.wt. increase (Figure 33). We will show that the arginase flux in arg-12 ure is negligible at total extractable ARG concentrations below 3 μmol . 100 mg^{-1} d.wt. (Section 4.3.7.3). If the essentially constant CIT and ARG pools in cultures CH 15 to 19 (Table 15) represent the final steady state values then their sum (of the mean values for the five cultures) plus the estimated ASA pool (from Barthelmess et al., 1974) and protein ARG flux (from Tateson, 1971) will equal the rate of CIT depletion. The summation below shows this to be essentially true.

We have:

$$\begin{aligned}
 \text{Flux in} &= \text{Flux out} \\
 &= F \times \text{CIT} + F \times \text{ASA} + F \times \text{ARG} + F \text{ protein ARG} \\
 &= (0.31 + 0.13 + 0.89 + 13.0) \mu\text{mol } 100 \text{ mg}^{-1} \\
 &\quad \text{d.wt. increase} \\
 &= 14.33 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. increase} \\
 &\approx \text{Rate of CIT depletion (} 15 \mu\text{mol. } 100 \text{ mg}^{-1} \\
 &\quad \text{d.wt. increase)}.
 \end{aligned}$$

Example 5

Although we can interpret with some confidence the plot of total CIT and ARG against weight for the data from Experiment XIV as showing a new steady state established towards the end of the experimental time course (see part (a) above and Figure 67) it would be reassuring to establish this to be true by an independent method. We can, in fact, use the "balance sheet" approach to ascertain whether or not the arg-12 ota ure system is at steady state at least for a biosynthetic flux to ORN equal to that characteristic of minimally grown ure. The calculation is as follows:

We know from the work of others (Tateson, 1971) and will show in Section 4.3.7.3 to this thesis that the arginase flux in ure on MIN medium or arg-12 ure at an equivalent ARG pool, is negligible. The OTCase flux in ure on MIN medium has also been shown to be negligible (Karlin et al., 1976).

Taking again the values for F ODCase from Karlin et al. (1976) and F prot ARG from Tateson (1971) as 2.2 and 13.0 $\mu\text{mol.}100 \text{ mg}^{-1} \text{ d.wt.}$ increase, respectively, we therefore have for ure on MIN medium:

Biosynthetic flux to ORN = Flux to expansion of ORN pool

+ ODCase flux + OTCase flux

$F \text{ bio ORN} = F \times \text{ORN} + F \text{ ODCase} + F \text{ OTCase}$

$= F \times \text{ORN} + F \text{ ODCase} + F \times \text{CIT} + F \times \text{ASA} + F \times \text{ARG} + F$

prot ARG

$= 0.67 + 2.2 + 0.20 + 0.13 + 2.49 + 13.0$

$= 18.69 \mu\text{mol.}100 \text{ mg}^{-1} \text{ d.wt. increase}$

The CIT and ARG pool values used above are the mean values from Experiments VIIb and IX (Tables 11 and 13, respectively). The ASA pool value is taken from Barthelmess et al., (1974).

Since the biosynthetic flux to ORN in arg-12 ota aga reduces to the sum of the flux to expansion of ORN and the ODCase flux, the ORN pool corresponding to a ure MIN flux (18.69-2.2) is 16.49 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ Extrapolating from Figure 70, this ORN pool corresponds to an ARG pool of 1.6 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ This is somewhat lower than the expected ARG pool of 2.49 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ on the assumption that the system was at steady state, i.e. the "proximal" flux had adjusted instantaneously to the "distal", falling ARG in Experiment XIV. This disparity is probably partly experimental; on the one hand, the ORN to ARG relation is very steep at the values of ARG concerned and, on the other, there is a large standard deviation (0.41) attached to the mean of the ARG pool values in ure on MIN medium. (The value for the ARG pool given by Barthelmess et al., (1974), based on a greater number of determinations than here is 2.08 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$). We will see in Section 4.3.7.2 that the fall in cytosolic ARG, i.e. the signal pool, appears to lead the decay in the total extractable ARG pool in arg-12 ota aga. On this basis, the ORN pool at a total extractable ARG pool of 2.49 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ would be higher than the 16.49 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ expected from the ure MIN calculation. That, if anything, the opposite is observed may be due to experimental error, or, if real, might be due to a degree of reversibility in the "proximal" moiety at high concentrations of ORN.

On balance, the evidence from the ARG to CIT relation (Figure 70), the change in rate of increase in total ARG (Figure 67 and part (a) above) and the comparison with ure MIN suggests that while not at a final steady state a quasi-steady state is approached in the tail end of Experiment XIV.

Extending the comparison of ure MIN to the arg-12 ure data from the "run down" experiments Xa and b we can show that the OTAase flux is substantially underestimated by the accumulation of PRO. Because the arginase flux is known to be negligible (Tateson, 1971 and see Section 4.3.7.3), we have for arg-12 ure, at an ARG pool of $2.49 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$:

Biosynthetic flux to ORN = Flux to expansion of ORN pool + ODCase flux + OTAase flux.

$$\begin{aligned} F \text{ bio ORN} &= F_{\text{LX}} \text{ ORN} + F \text{ ODCase} + F \text{ OTAase} \\ &= F \times \text{ORN} + F \text{ ODCase} + (F \times \text{PRO } \underline{\text{arg-12 ure}} - F \times \text{PRO } \underline{\text{ure}}) \\ &= 1.30 + 2.2 + (0.95 - 0.31) \\ &= 4.14 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. increase} \end{aligned}$$

The arg-12 ure data are extrapolated from Figures 36 and 37. The value for $F \times \text{PRO } \underline{\text{ure}}$ is the mean from Experiments VIIb and IX (Tables 11 and 13 respectively).

The "deficit" of $14.55 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. increase}$ in the above calculation of the biosynthetic flux to ORN in arg-12 ure can only be accounted for by the increase in PRO over the ure value seriously underestimating the OTAase flux. In fact, we can estimate that in arg-12 ure under these pool conditions the OTAase flux comprises about 80% of the total flux to ORN of which only about 1% is seen as an increase in the PRO pool. The bulk of this flux must be lost in GLU formation.

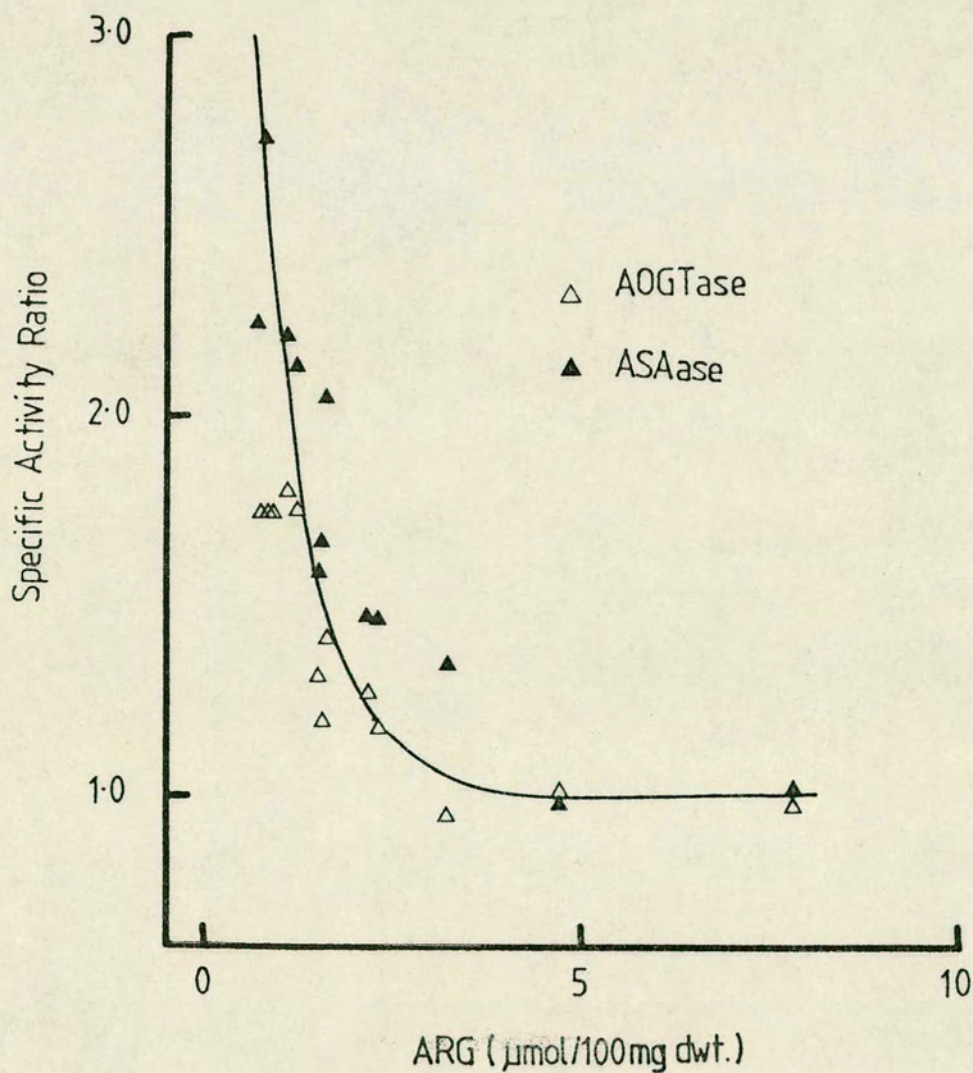
Two conclusions follow from the demonstration above, that, whereas in ure on MIN the OTAase flux is negligible, the corresponding flux in arg-12 ure at an equivalent ARG pool is very large. Firstly, the origin and subcellular distribution of ORN

is a decisive factor in determining the subsequent metabolic fate of ORN. Recalling from Section 1.3.2.1, it has been shown that in wild-type on minimal medium over 90% of the total extractable ORN is sequestered to the metabolically inert vesicle with less than 5% held in the mitochondria and less than 1% in the cytosol (Weiss, 1973 and Karlin *et al.*, 1976). Under these conditions the concentration of cytosolic ORN is probably less than the K_m for OTAase which accounts for the negligible OTAase flux despite a large total extractable ORN concentration (Weiss and Davis, 1973). In wild-type biosynthetically derived ORN formed in the mitochondria is predominantly channeled by mitochondrially localised OTCase to ARG biosynthesis (Bowman and Davis, 1977a and b). The arg-12 mutation prevents the flux of biosynthetically derived ORN to ARG. Consequently the concentration of ORN in the mitochondria will increase and will pass (actively or by diffusion) into the cytosol where it will accumulate and pass (probably actively) into the vesicle. The net effect will be an increase in the total extractable concentration of ORN and, in particular, the cytosolic concentration, compared with that characteristic of minimally-grown wild-type cultures. In fact, most of the (cytosolic) ORN is shown above to be catabolised by cytosolic OTAase. The second conclusion, therefore, is that the OTAase flux is largely determined by mass action; the slightly higher specific activity of OTAase in arg-12 ure under the "run down condition" compared with ure on minimal medium at equivalent ARG pools (Figure 45) can only account for a small proportion of the inferred increase in flux.

Figure 68: Experiment XIV: the relation between ARG and the specific activity of AOGTase and ASAase.

Data from Table 19, Appendix III.

See accompanying text for details.



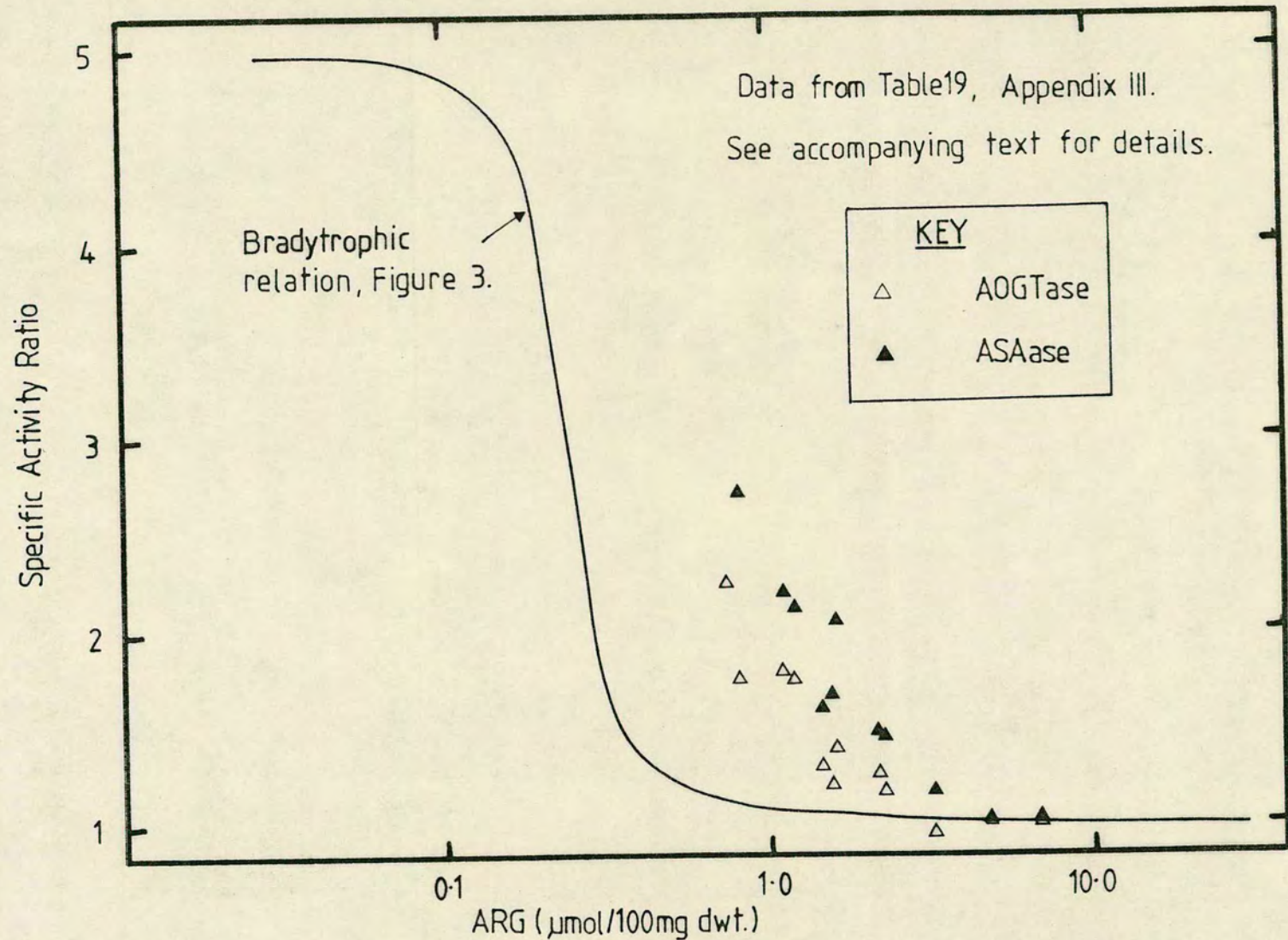
The conclusion that the origin and sub cellular locality of ORN largely determines its subsequent metabolic fate is also shown by the ORN to ARG relation in arg-12 ure (Figure 36). We will show that there is no significant increase in the biosynthetic flux to ORN for a fall in ARG from about 20 to 13 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ (Section 4.3.7.3). However, there is a significant drop in the arginase flux to ORN over this range (Flint, 1977). That no significant drop in the total extractable ORN pool in arg-12 ure is observed over this range must mean that ARG-derived ORN formed in the cytosol is predominantly channelled by cytosolic OTAase to GSA and not sequestered to the vesicle or mitochondria. This interpretation is in complete accord with the tracer studies by Karlin et al (1976) and Bowman and Davis (1978a and b) of the fate of biosynthetically derived, exogenously derived and ARG derived ORN.

4.3.7.2

The derepression of AOGTase and ASAase in arg-12 ota ara

The specific activity of AOGTase and ASAase in arg-12 ota ara were determined only in cultures from Experiment XIV since only there were ARG pools within the "derepression" range established. We have already seen from the time course of events (Figure 62) that these two specific activities increased substantially and coordinately with their rise coinciding with the fall in ARG pool to the "expected" range (Figure 61). The relation between the specific activities and the ARG pool is shown in Figure 68. (The specific activities have been expressed as the ratio over the mean

Figure 69: The effect of the HIS:CIT "run down" condition on derepression in arg-12 ota aga.



for cultures CH + H1 and 2 to show the degree of derepression). The data describe a typical derepression curve. However, as for the 'run down' experiment with the arg-12 ure strain (Experiment Kb) the derepression appears to start at ARG pools higher than observed either under "steady state" HIS:CIT conditions or in minimally grown bradytrophic strains (Figure 40). As before, this is seen more clearly when the specific activity ratios are plotted against the log of the ARG pool with the derepression curve of Barthelmess et al (1974) superimposed, as shown in Figure 69.

In discussing the shift observed under the arg-12 ure "run down condition" (Section 4.3.3.2) we discarded the possibility that the lag in arginase specific activity is the cause since, as will be shown in Section 4.3.7.3, no arginase flux is detectable in the "derepression range" (Figure 72). This is confirmed by the observation that a shift is also seen in the arg-12 ots arg "run down condition", where no arginase activity is present. As before, we must conclude that the rate of removal of cytosolic ARG is greater than the rate of equilibration of ARG between the vesicular and cytosolic compartments. The fact that the shift in arg-12 ots arg is greater than observed in arg-12 ure suggests that ORN (and, presumably HIS also) does effect the rate of equilibration of ARG between the compartments. This follows from the fact that, if anything, the flux into ARG is higher in arg-12 ots arg (4:1 HIS:CIT ratio) than in arg-12 ure (5:1 HIS:CIT). However, the total extractable concentration of ORN is much higher in the arg-12 ots arg than the arg-12 ure strain before and after the "derepression range" is reached.

The derepression of AOGTase must result in a proportional increase in the biosynthetic flux to ORN if, as is reasonably assumed, derepression is coordinate throughout the "proximal" as well as "distal" enzymes. Any flux increase over and above that attributable to derepression must be due to deinhibition. The dissociation of the derepression and deinhibition effects of ARG will be described and discussed in the following and final section.

4.3.7.3

The control of flux through the arginine pathway;
calculations and conclusions.

We have already discussed in some depth the relationships between pools and between enzymes and pools described by the arg-12 ure under both the "run down" and "run up" condition (Sections 4.3.3 and 4.3.5 respectively). Many of the conclusions drawn from those relations were, of necessity, tentative because of the uncertainties attached to the determination of the arginase and OTAase fluxes and their relative contributions to the accumulation of ORN. Now that we have data for the arg-12 ota aga strain complementary to the arg-12 ure "run down" condition many of these interpretative difficulties can be overcome. Both the arginase and the OTAase flux in the arg-12 ure can be estimated from a comparison of the ARG to CIT and ORN to ARG pool relations already described for arg-12 ure (Figures 25 and 36) with those generated by the arg-12 ota aga "run down" data. The complete metabolic dissociation of the "proximal" and "distal" moieties of the pathway in the arg-12 ota aga permits a direct assessment to be made of the ARG effect on the biosynthetic flux to ORN through deinhibition and derepression. The relative

Figure 70: The "run down" relation between CIT and ARG
in arg-12 ota aga.

Data from Experiments XII(∇), XIII(\blacksquare) and XIV(\blacktriangledown), Tables 17, 18 and 19,
Appendix III. See accompanying text for details.

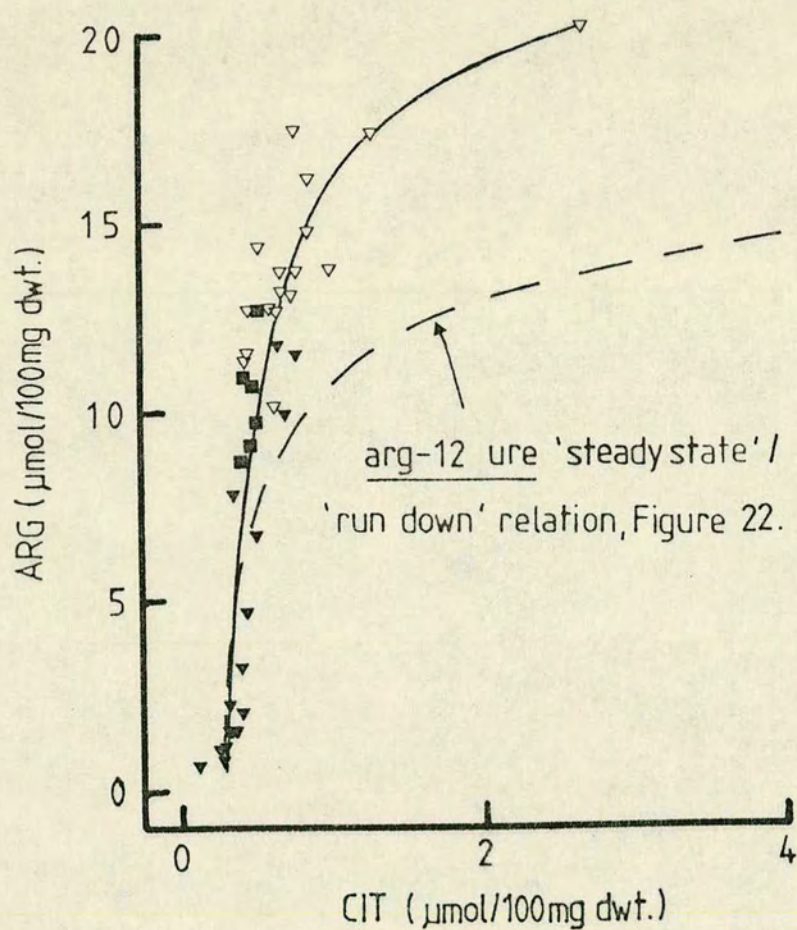


Figure 71: The "run down" relation between CIT and FxARG in arg-12 ota aga.

Data from Experiments XII (∇), XIII (\blacksquare) and XIV (\blacktriangledown), Tables 17, 18 and 19, Appendix III. See accompanying text for details.

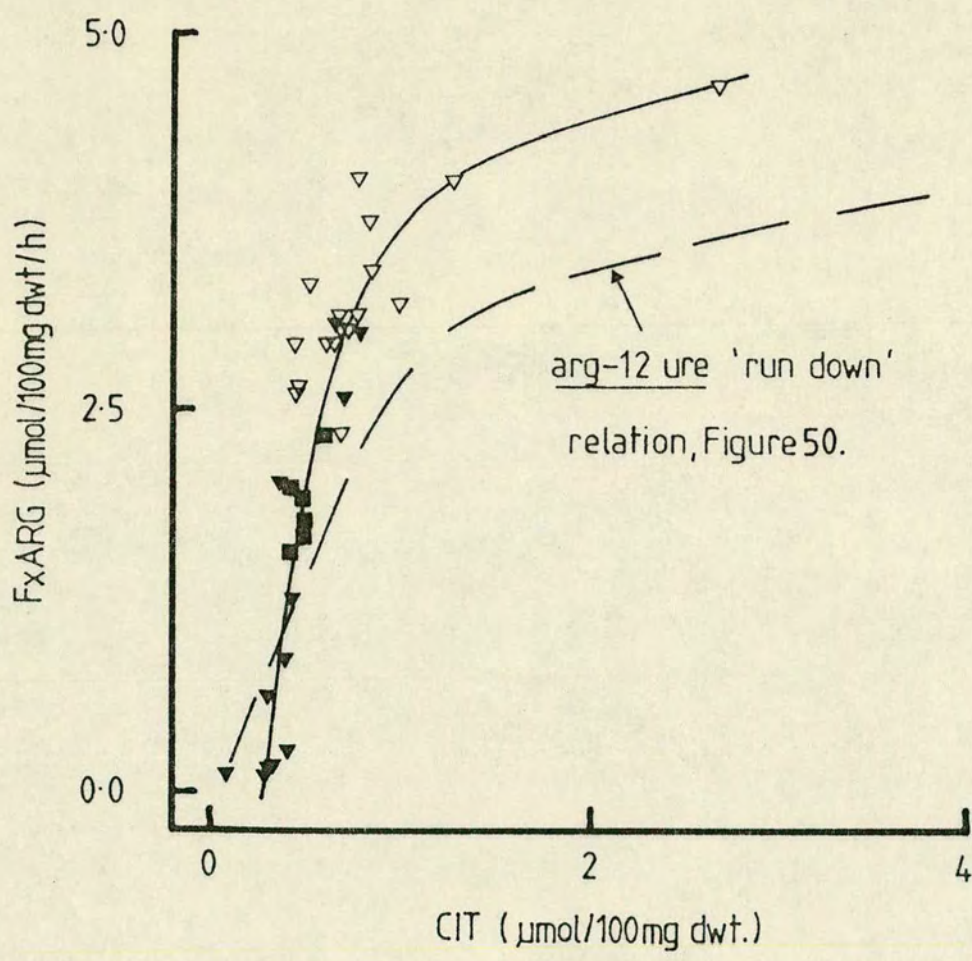
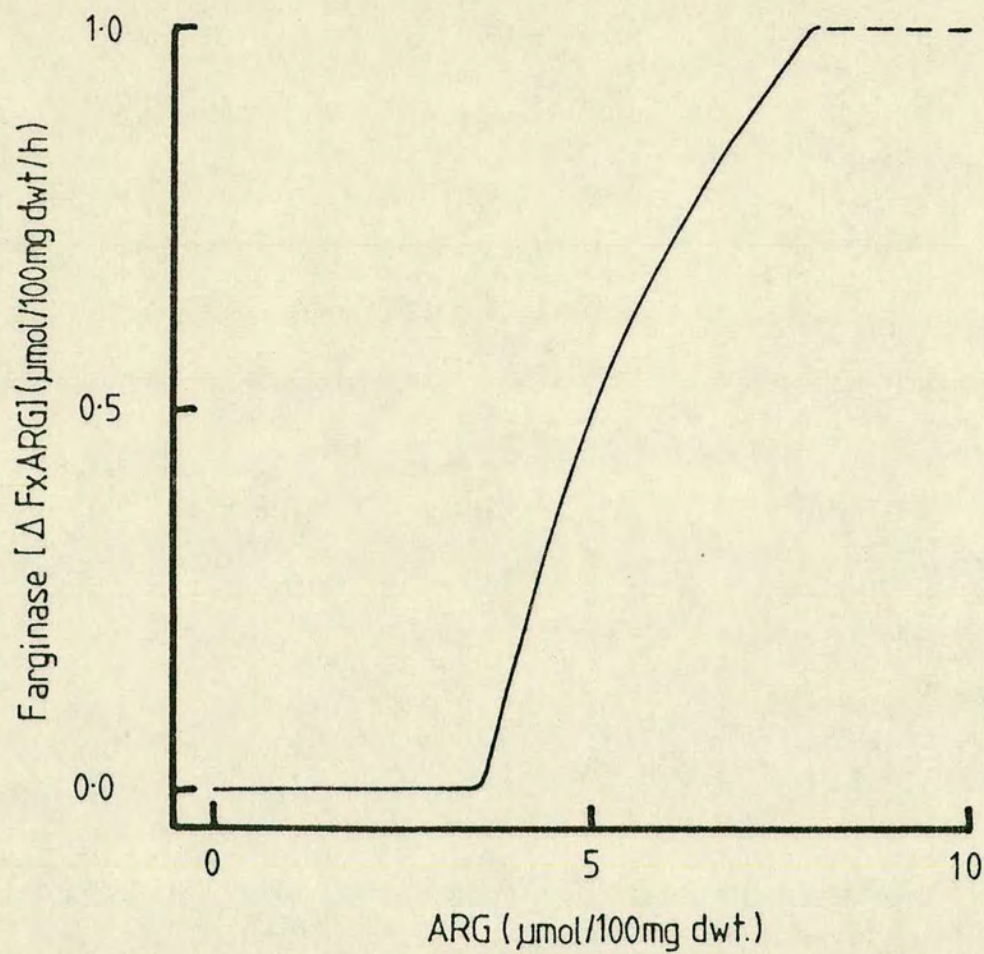


Figure 72: The relation between ARG and the arginase flux in arg-12 ure.

Plot constructed from the data shown in Figures 71 and 35 .

See accompanying text for details.



kinetics of deinhibition and derepression can ^{be dissociated} by consideration of the prevailing degree of AOGTase derepression.

a) Comparison of the ARG to CIT relation between the arg-12 ota aga and arg-12 ure strains.

The ARG to CIT pool relation generated by the data from Experiments XII to XIV employing the arg-12 ota aga strain ^{is} shown in Figure 70 with the arg-12 ure relation, taken from Figure 22, superimposed. The figure shows that in arg-12 ota aga the ARG response to CIT is greater than in the arg-12 ure strain, but that the form of the relationship is essentially the same, showing the now familiar saturation at quite low CIT values.

There is no evidence for either SYNase or ASAase being subject to inhibition or activation (Davis et al, 1978a), only derepression at low ARG pools (Figure 3). Since both arg-12 ure and the arg-12 ota aga systems have been shown to be at a quasi-steady state under the "run down condition", the flux to ARG at identical CIT pools can be assumed to be the same in both strains. The difference in flux to expansion of ARG ($F \times \text{ARG}$) between arg-12 ota aga and arg-12 ure at a given CIT concentration must equal the arginase flux in arg-12 ure at the value the ARG pool has. The $F \times \text{ARG}$ to CIT relation described by the arg-12 ota aga data is shown in Figure 71, with the arg-12 ure relation from Figure 50 superimposed. We can now construct the relationship between the arginase flux and the corresponding ARG pool in the arg-12 ure (extrapolated from Figure 35), as described above. The result is shown in Figure 72. The figure shows that at ARG concentrations less than $3.5 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ the arginase flux is a very small proportion of the ARG expansion flux and therefore a negligible flux out of ARG. At ARG concentrations

between 3.5 and 8.0 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ there is an approximately proportional increase in arginase flux. This estimation of the arginase flux agrees fairly closely with the direct determination of the flux by Flint (1977) from the rates of urea accumulations at different steady state ARG pools. (Flint (1977) determined an arginase flux of 1 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.h}^{-1}$ at an ARG pool of about 10 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ In the present case the same flux was estimated at an ARG pool of 8 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$)

The flux to pool relation will be due in part to (de)-induction of arginase enzyme and feedback inhibition by ORN. However, for a fall in ARG from 7.5 to 5.0 $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ the specific activity of arginase falls by only 10% (Figure 42) whereas the arginase flux falls by 50%. Furthermore, there is a substantial arginase specific activity at ARG concentrations where no arginase flux is detectable. Although feedback inhibition of ORN on arginase will also tend to reduce the arginase flux as the ARG pool falls most of the change in arginase flux occurs in the absence of significant changes in the ORN pool (Figure 36).

It follows that the dominant effect on the arginase flux must be mass action. The same conclusion was drawn from the ARG to CIT relation under the "run up" condition (Section 4.3.5.2, Figure 49). This conclusion is in accord with the finding that the K_m for ARG of arginase determined in vitro is less than the estimated cytosolic concentration of ARG available in minimally grown wild-type (Subramanian et al., 1973; Weiss and Davis, 1973); the arginase flux will therefore be dependent on the cytosolic ARG concentration over a considerable range above and below the wild-type level. However, this finding cannot explain the fact that

no arginase flux is detectable in minimally grown wild-type (Davis et al, 1978a); the concentration of available ARG would have to be effectively zero and certainly considerably smaller than the estimated cytosolic concentration for there to be no detectable catalysis purely on kinetic grounds. Assuming that the estimates of the cytosolic ARG concentration are of the right order of magnitude so far as the concentration which the enzyme "sees" is concerned this apparent inconsistency can only be explained by the kinetic behaviour of arginase in vivo being radically different from the Mn^{++} activated enzyme used for the K_m determination in vitro. This is almost certainly true since "native" arginase has a substantially different kinetic behaviour, with much lower activity and greater susceptibility to feedback inhibition by ORN, than the Mn^{++} activated enzyme (Davis, et al, 1978a, see also 2.2.7.3).

There appears from Figure 72 to be a saturation of the arginase flux at ARG values above about $8.0 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ This contrasts with the findings of Flint (1977) which showed the arginase flux to increase continually with ARG pool expansion. The apparent approach to an asymptote observed here may not be real, being based only on a single datum point. Obviously, it would be desirable to generate a large number of points covering the range of CIT values above $1 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ in the arg-12 ota aga strain and thus resolve (or confirm) this disparity between the present work and that of Flint (1977).

b) Comparison of the ORN to ARG relation between the
arg-12 ota aga and arg-12 ure

The ORN to ARG relation generated by the data from Experiments XII to XIV employing the arg-12 ota aga strain, is shown

Figure 73: The "run down" relation between ARG and ORN in arg-12 ota aga.

Data from Experiments XII(∇), XIII(\blacksquare) and XIV(\blacktriangledown), Tables 17, 18 and 19, Appendix III. See accompanying text for details.

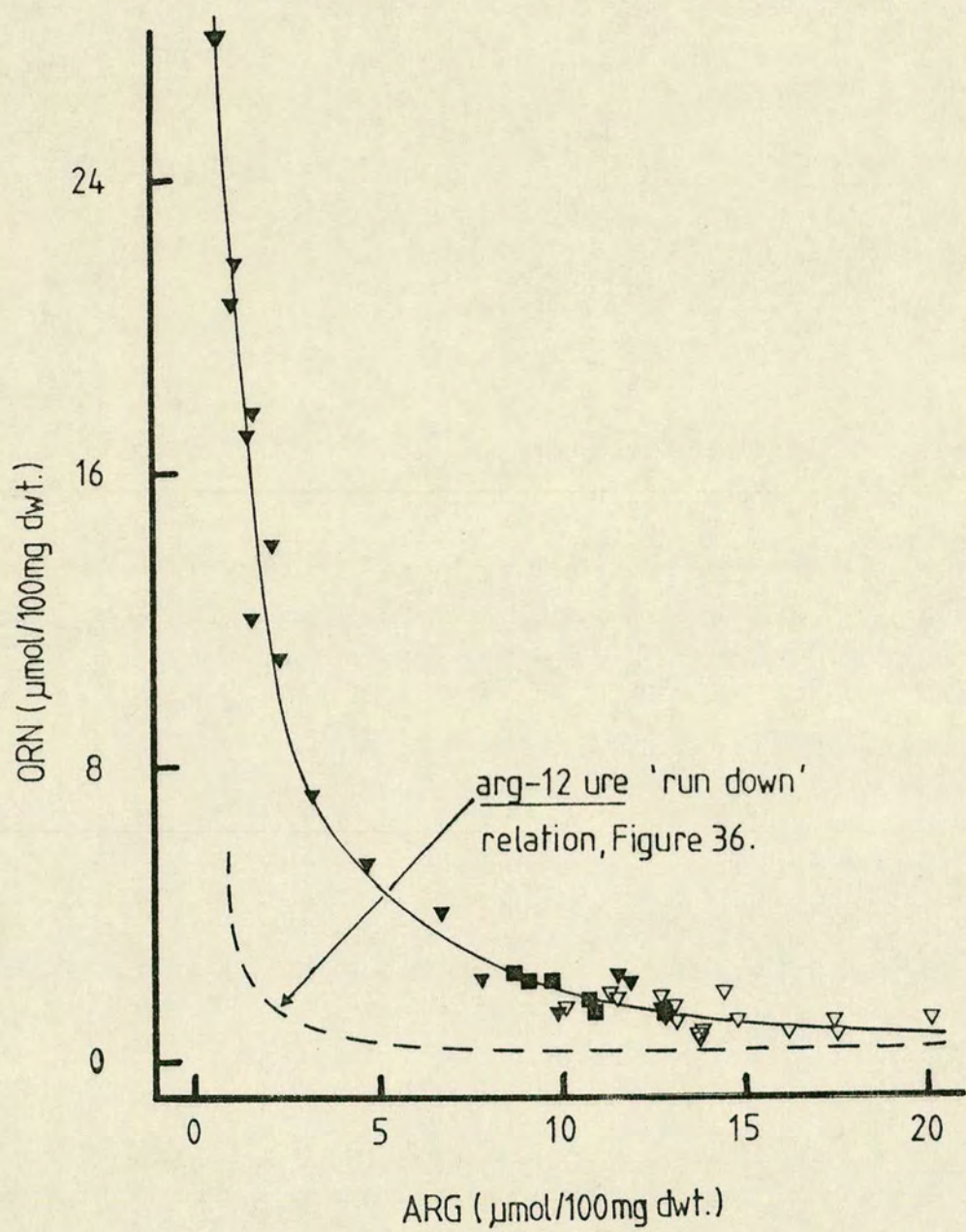


Figure 74: The "run down" relation between ARG and FxORN in arg-12 ota aga.

Data from Experiments XII (∇), XIII (\blacksquare) and XIV (\blacktriangledown), Tables 17, 18 and 19, Appendix III. See accompanying text for details.

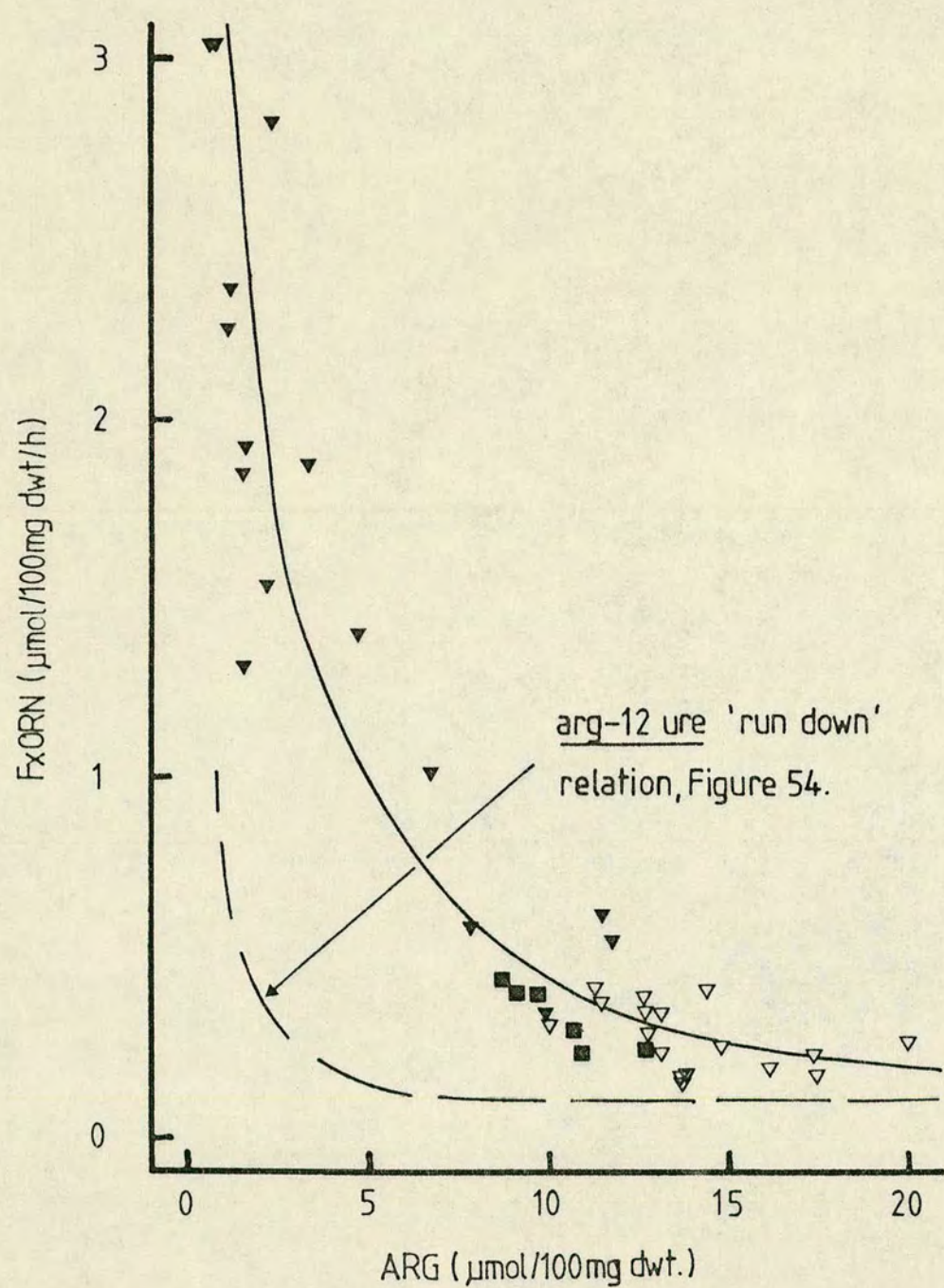
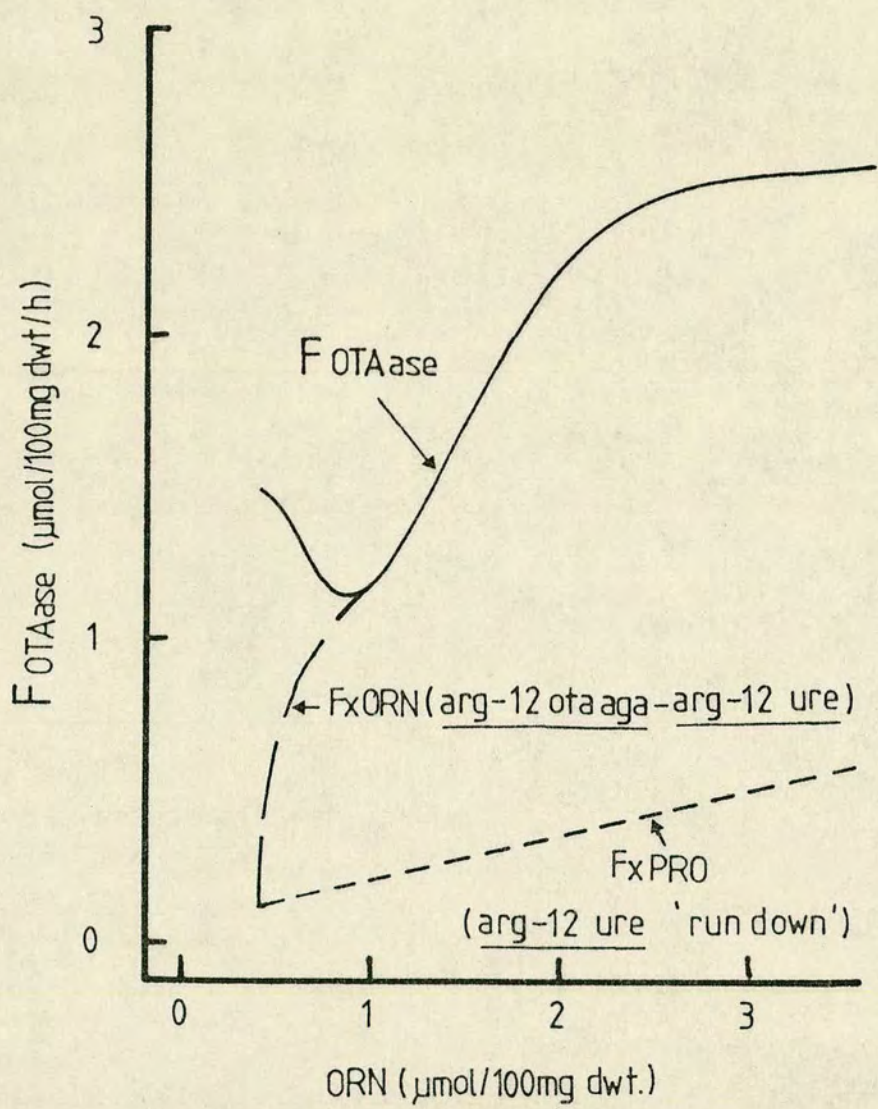


Figure 75: The relation between ORN and the OTAase flux in arg-12 ure.

Plot constructed from the data shown in Figures 36, 37 and 74.
See accompanying text for details.



in Figure 73 with the arg-12 ure relation taken from Figure 36 superimposed. What was described as a dramatic response of ORN to ARG in arg-12 ure is almost completely over-shadowed by the response observed in the arg-12 ota aga strain. Uncomplicated by the contribution of the arginase (positive) and OTAase (negative) fluxes present in arg-12 ure, this ORN response in arg-12 ota aga reflects directly the effector role of ARG on the biosynthetic flux through the "proximal" moiety. The dissection and quantitation of the ARG effect through the mechanisms of deinhibition and derepression will be dealt with shortly. First of all we can see how, from a comparison of the ORN pool responses between the two strains, the OTAase flux in arg-12 ure can be estimated.

If we make the reasonable assumptions that i) the biosynthetic flux to ORN is the same in both strains at equal ARG pools, and ii) the ODCase flux to polyamine synthesis is a universal constant, as assumed in all previous calculations, then the difference between the two strains in the $F \times \text{ORN}$ at equal ARG pools must equal the difference between the sum of the OTAase and arginase fluxes in arg-12 ure. The relationship between the $F \times \text{ORN}$ and the ARG pool for the arg-12 ota aga data is shown in Figure 74, with the arg-12 ure relation from Figure 54 superimposed. We know what the arginase component of the $F \times \text{ORN}$ difference is from the comparison of the two ARG to CIT relations.

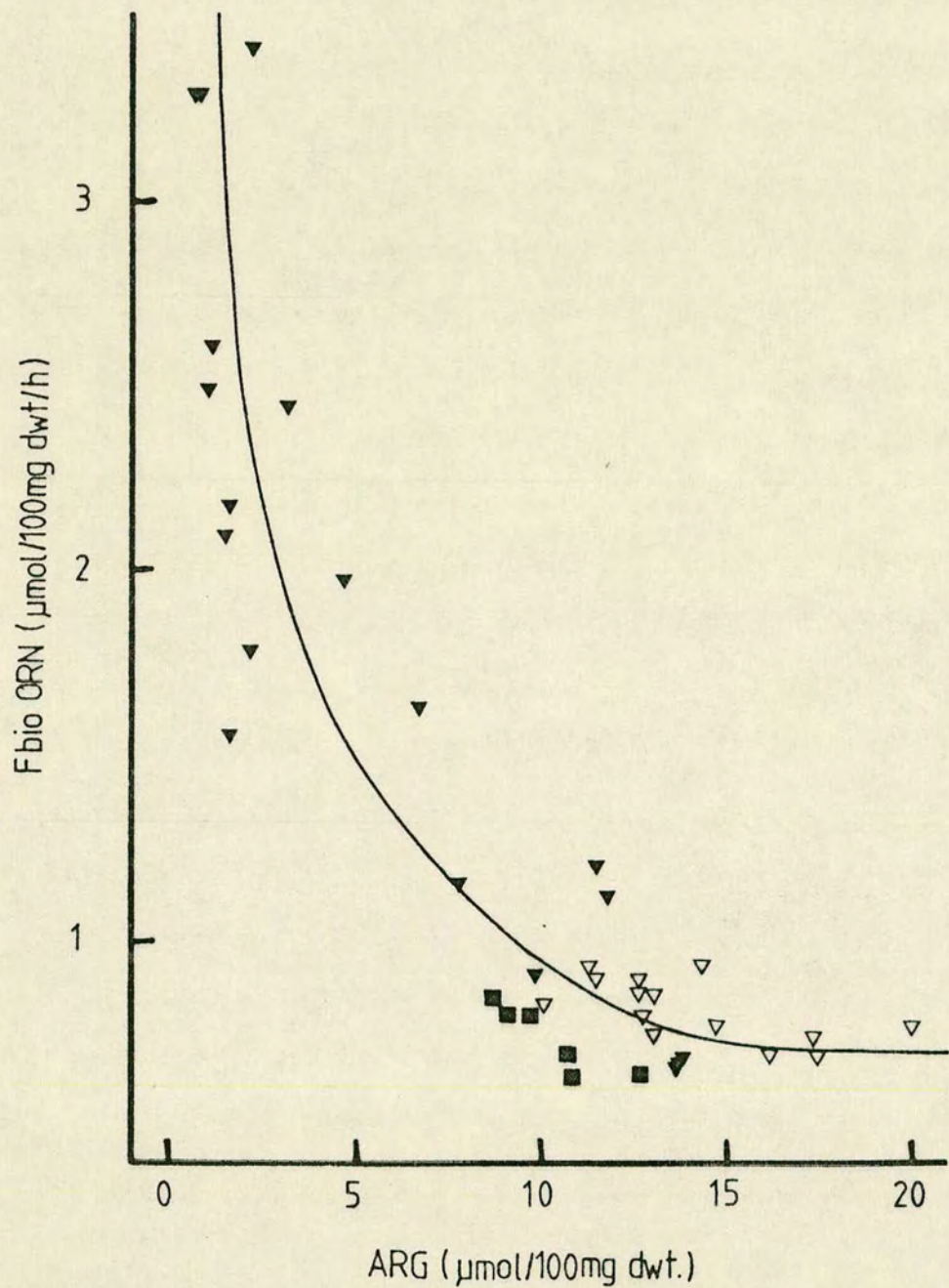
By subtraction we can obtain an estimate for the OTAase flux. This is shown plotted against the corresponding arg-12 ure ORN pool in Figure 75.

Also shown in the figure is the relationship between the strain difference in F x ORN and the corresponding arg-12 ure ORN pool. The contribution of ARG to ORN via arginase is only detectable at ARG pools greater than $3.5 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ (Figure 72). Therefore, the F x ORN and F OTAase relationships only diverge at ORN pools less than $1 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ (Figure 36).

The F x PRO to ORN relationship for Experiments Xa and b (from Figure 37) is superimposed on Figure 75 for comparison with the above method of F OTAase estimation. ^{Clearly, the two methods give very different answers for the OTAase flux.} Although the calculation of the OTAase flux from the difference in F x ORN between the arg-12 ota aga and arg-12 ure strains involves extrapolation from several different pool to pool relations this method is believed to yield a fairly reliable estimate since the intrinsic assumptions made in the calculation are well founded. In contrast, there is good reason to expect the accumulation of PRO in arg-12 ure to underestimate the OTAase flux. This has already been discussed in Sections 4.3.3.3 and 4.3.7.1. The comparison of the two methods strongly suggests that the majority of the OTAase flux is not seen as accumulated PRO but is "lost" presumably in GLU formation. We may presume therefore that under our condition the majority of biosynthetically derived cytosolic ORN which is catabolised by OTAase is then oxidised to GLU rather than reduced to PRO. The exact opposite is found to occur in wild-type Neurospora at low levels of cytosolic ORN (Davis et al, 1978a). The reason for this change in the relative rates of GLU and PRO formation with ORN concentration is not known but may be formally explained if the "Km" for GLU formation is much higher than for PRO formation; the rate of GSA formation we know to be largely determined by mass action (Section 4.3.3.3). Certainly

Figure 76: The relation between ARG and the biosynthetic flux to ORN.

Data from Experiments XII (∇), XIII (\blacksquare) and XIV (\blacktriangledown), Tables 17, 18 and 19, Appendix III. See accompanying text for details.



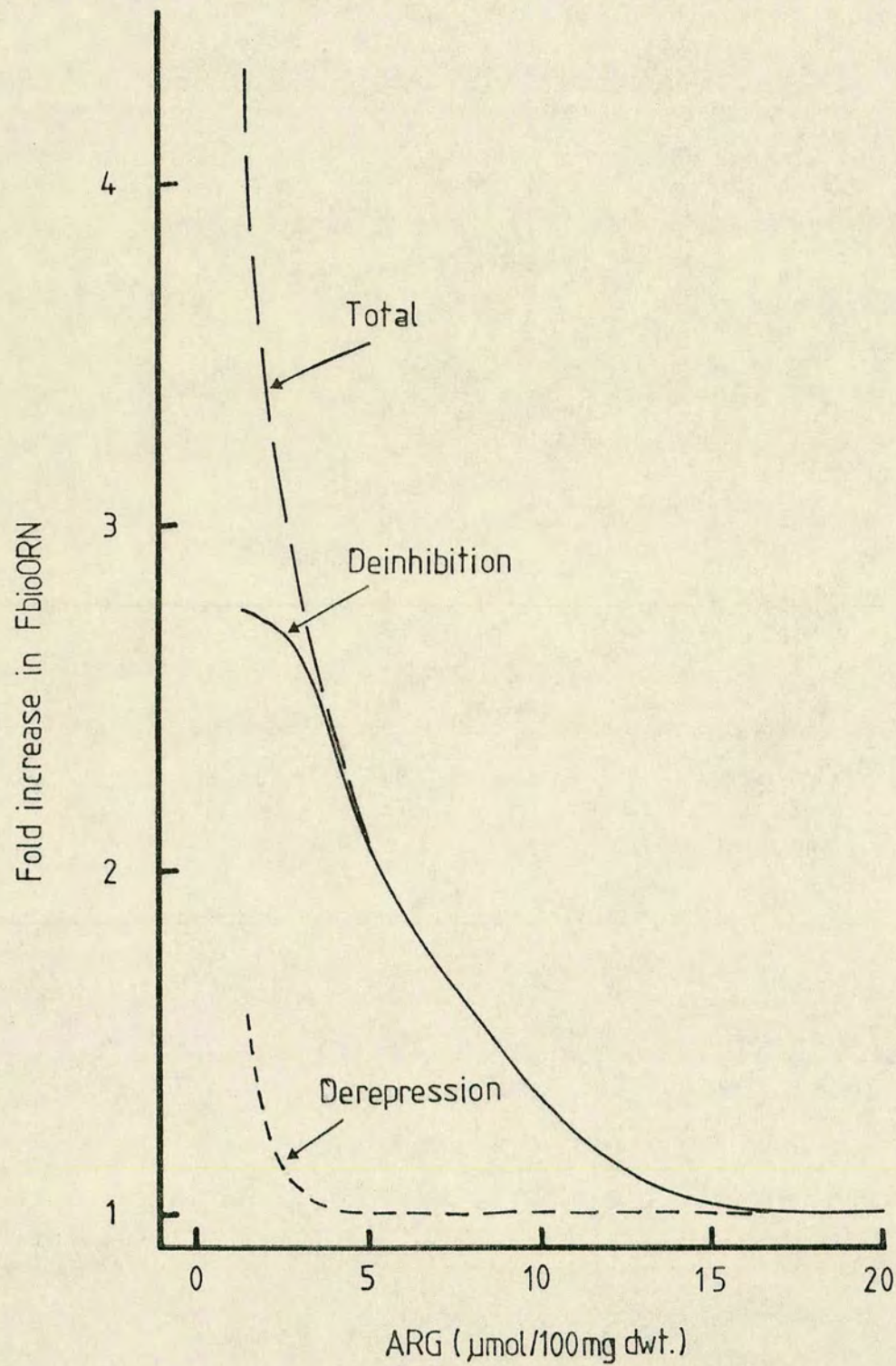
the PRO to CIT relation under the "run down" condition in arg-12 ure (Figure 37) does suggest that the GSA to PRO flux saturates at relatively low ORN concentrations.

Finally, we can now consider what the relative contributions of deinhibition and derepression are to the control of flux through the pathway. The analysis of the control of biosynthetic flux to ORN in the arg-12 ure strain was severely restricted by the unknown effect of the arginase and OTCase fluxes on the level of ORN accumulated. In the arg-12 ota aga strain the biosynthetic flux to ORN reduces to the sum of only two components. Firstly, there is the flux to expansion of the ORN pool itself and, secondly, there is the flux to polyamine synthesis via ODCase. We know from the work of Karlin et al (1976) that the steady state pool of polyamines remains substantially constant with a value of $2.2 \mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ under varying conditions of ARG and ORN pools. We can therefore express the biosynthetic flux to ORN ($F_{\text{bio ORN}}$) in arg-12 ota aga as $k(\text{ORN} + 2.2)$, where k is the growth rate constant and ORN the observed quasi-steady state pool. The flux is then in terms of $\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt. h}^{-1}$.

The relationship between the ARG pool and $F_{\text{bio ORN}}$ for the arg-12 ota aga data is shown in Figure 76. From this figure we can calculate the fold increase in $F_{\text{bio ORN}}$ with falling ARG pool, taking the value of 0.7 as the fully inhibited and repressed flux. If we make the reasonable assumption of co-ordinate derepression in the "proximal" moiety the fold increase in $F_{\text{bio ORN}}$ attributable to derepression will equal the specific activity ratio (SAR) at the corresponding ARG pool (Figure 68). Extrapolating from Figures 68

Figure 77: The relation between ARG and the fold increase in the biosynthetic flux to ORN; the kinetics of de/inhibition.

Plot constructed from the data shown in Figures 76 and 68 .
See accompanying text for details.



and 76, we can now determine the kinetics of deinhibition with falling ARG pool as the ratio of the fold increase in F bio ORN over the SAR of the biosynthetic enzymes. This calculation yields the plot shown in Figure 77. Also indicated in the figure are the fold increase in F bio ORN and the kinetics of derepression.

The figure shows that the mechanisms of deinhibition and derepression operate over substantially different ranges of ARG pool. The kinetics of deinhibition describe a sigmoidal function, indicative of an allosteric transition, with the mechanism affecting a flux response almost as soon as the ARG pool starts to fall from CIT grown values, reaching a maximum slope at an ARG pool of about $4 \mu \text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$ and beginning to plateau as the ARG pool falls into the derepression range. As seen previously (Figures 40 and 69) and shown by this graph, the derepression mechanism operates at a much lower range of ARG pools.

It will be noticed that at all levels of ARG pool generated in arg-12 ota aga the fold increase in flux attributable to deinhibition is greater than that accounted for by derepression. However, the data indicate that deinhibition can affect at most about ^a2.75-fold flux increase whereas 5-fold coordinate derepression of the biosynthetic enzymes is reported (see Figure 3). We have seen, though, that the "controlling" ranges for the two mechanisms barely overlap and therefore comparison of their maximal contributions to flux increase is largely irrelevant. However, an important and highly relevant comparison is of their relative effectiveness at responding to ^achange in ARG with a change in flux, i.e. their slopes at particular concentrations of ARG. In this regard, derepression, with clearly the steeper response curve, is the more efficacious mechanism.

In the arg-12 mutants the effect of deinhibition is only "felt" by the "proximal" part of the pathway, whereas derepression also acts directly upon the flux through the "distal" part. The effectiveness of the derepression mechanism is shown by the fact that the lowest, quasi-steady state ARG pool achieved was in the "middle" of the "controlling" range, i.e. the point of steepest slope on the response curve (Figures 16, 39 and 68); the derepression mechanism effectively buffers the ARG pool against further change in system influx.

The dramatic response in *F* bio ORN to modulation of the ARG pool (Figure 76) argues strongly that both deinhibition and derepression are important and effective mechanisms for the control of flux through the ARG pathway. However, by extension of the argument developed by Barthelmess et al (1974), "if derepression or deinhibition were the principal mechanism for "controlling" the level of the pathway end product, arginine, the wild-type level would be found in the "middle" of the relevant control range. The level is in fact between the two control ranges, and therefore, the feedback loop of signal on enzyme amount or activity is not operating significantly when wild-type is grown on Vogel's minimal medium.

The steady state level which arginine settles to is, therefore more a function of the metabolic interactions inherent in the pathway than the effector role of arginine. It is, therefore, not exclusively "controlled" (Kacser and Burns, 1973) by either of the mechanisms that might be thought to have been evolved for that purpose".

This argument is, of course, limited to the steady state characteristic of a particular, albeit "minimal", defined medium.

It does not preclude the effective operation of both mechanisms in the maintenance of balanced growth under conditions of a possibly, highly variable environment in the wild particularly with respect to the availability of carbon, nitrogen and sulphur sources.

In the final analysis, we can see that the behaviour of our experimental system closely matches the certainties which arise from an algebraic analysis of steady state behaviour (Kacser and Burns, 1973 and 1979). We have seen that the metabolic steady state and the fluxes which generate it are systemic properties which can only be analysed and understood by the method of modulation.

The kinetic properties of enzymes, their subcellular distribution and that of their substrates, products and other effectors, pool expansion, mass action and saturation, derepression, deinduction and deinhibition; all these system components have been shown to affect, to varying degrees at different metabolic steady states, the control of flux through the arginine pathway in Neurospora crassa.

APPENDIX I

GENETIC SYSTEMS OF METABOLIC REGULATION IN FUNGI

In this appendix some of the regulatory genetic systems in fungi of particular interest to the author are described for comparison with the organisation and regulation of the arginine pathway in Neurospora as outlined in Section 1.3.2 and analysed in the experiments described in the Results and Discussion.

(a) The regulation of arginine metabolism in Saccharomyces cerevisiae

The regulation of arginine metabolism in the yeast, Saccharomyces cerevisiae, has been extensively investigated by Wiame and co-workers (see Wiame, 1971 for a review of early work and ideas and this text for specific references). It is of particular interest to compare the regulatory mechanisms demonstrable in this fungus with those described for Neurospora crassa (see Section 1.3.2).

As in Neurospora, synthesis of several of the enzymes associated with arginine metabolism in Saccharomyces is repressible by arginine; but, unlike the situation in Neurospora, synthesis of the Saccharomyces enzymes is not fully repressed during growth in minimal medium; repression occurs upon addition of arginine to the growth medium. The range of enzyme activities in the arginine synthesis pathway of Saccharomyces is an order of magnitude higher than in Neurospora (Wiame, 1971). Three separate regulatory circuits are involved in repression of synthesis of these enzymes in Saccharomyces, one general and two specific to the enzymes involved

in arginine synthesis. The "general" regulatory system affects synthesis of enzymes involved in arginine synthesis and in metabolism of several other aminoacids (Delforge et al, 1975; Wolfner et al, 1975). One of the "specific" regulatory systems controls the synthesis of carbamyl phosphate synthetase specific to the arginine pathway (Thuriaux et al, 1972). S. cerevisiae, like N. crassa, has an arginine pathway specific CAP synthetase, coded by two unlinked genes, cpaI and cpaII. Unlike N. crassa, CAP formed by these two enzymes is not channelled in S. cerevisiae; the CAP formed by each synthetase is available to both arginine and pyrimidine biosynthesis (Lacroute et al, 1965). The synthesis of the CAP synthetase specific to the arginine pathway is regulated by a negative type of control. The cpaIO mutations, closely linked to the structural gene cpaI, are cis-dominant and have the properties of 'operator' mutations. The cpaR mutations are unlinked to the structural genes; their phenotypic effects are consistent with their forming an inactive 'repressor' of CAP synthetase (Thuriaux et al, 1972). The second "specific" circuit controls the synthesis of most of the other anabolic enzymes. A specific aporepressor for this circuit has been identified by its deficiency in arg-R mutant strains. The products of three unlinked loci, argRI, argRII and argRIII, are necessary for the formation of an active aporepressor (Bechet et al, 1970). This aporepressor is distinct from that identified for CAP synthetase synthesis (Thuriaux et al, 1972). A negative mode of control has again been defined through the isolation of an 'operator' type mutation. These mutants, tightly linked to the structural gene for OTCase, are cis-dominant and result in the specific constitutive synthesis of OTCase (Messenguy, 1976).

At least as far as ornithine transcarbamylase (OTCase) activity is concerned, both "specific" and "general" control circuits appear to act at the level of transcription (Messenguy and Cooper, 1977).

A recent study (Minet et al., in press) provides evidence for the co-ordinate expression, probably determined at the level of transcription, of two sequentially functioning enzymes in the arginine synthetic pathway, acetylglutamate kinase (AGKase) and acetylglutamyl-phosphate reductase (AGP reductase). Evidence from fine structure mapping, complementation tests and the isolation of nonsense mutants exhibiting polar effects, suggests that the cluster is transcribed as a single unit and translated, starting at a single initiation site, as a single polypeptide which is subsequently cut into two enzymes; alternatively, transcription may initiate at one or both of two initiation sites, occurring at the beginning of each gene.

The control of the synthesis of the catabolic enzymes in S.cerevisiae is complex. Arginase and OTAase activity are induced by arginine and glutamate and repressed by ammonia (Wiame, 1971; Middlehoven, 1964). (Arginase activity is also competitively inhibited by ornithine in vitro and possibly repressed by urea in vivo (Chan and Cossins, 1972)). Control of enzyme synthesis is mediated through at least three different regulatory circuits. The argR mutants previously described as leading to derepression of the anabolic enzymes also result in the non-induction of arginase and OTAase by arginine; consequently argR mutants are unable to grow on ORN, CIT or ARG as sole N source (Wiame, 1971). Taking advantage of this fact, 'operator' like mutants for both the OTAase (cargBO)

and arginase (carA0) structural genes were selected from argR mutants by their ability to grow on ORN, for example, as sole N source (Wiame, 1971). As expected, the selection technique also revealed mutants, designated carcR, unlinked to the structural genes which lead to the constitutive synthesis of high levels of arginase and OTCase, presumably through the formation of an inactive repressor protein (Wiame, 1971). Arginase is also one of the catabolic enzymes subject to ammonia repression in S.cerevisiae (Dubois et al, 1973 and 1974) (see following section).

In addition to these three mechanisms for the control of arginase synthesis can be added a possible control of activity in vivo through competitive inhibition by ORN, as demonstrated in vitro by Chan and Cossins (1972).

As well as being subject to a complex hierarchy of regulatory mechanisms, arginase itself is a regulatory protein (Wiame, 1971). Bechet and Wiame (1965) observed that ornithine transcarbamylase (OTCase) activity decayed on addition of arginine to cultures of S.cerevisiae growing on minimal medium. The decrease in activity was shown to be due to the reversible inactivation of OTCase activity through the reversible binding of the OTCase enzyme with another protein. The binding protein was later shown to be arginase (Messenguy and Wiame, 1969). The experimental evidence suggests the following mechanism for the arginase-OTCase activities.

In S.cerevisiae OTCase has two binding sites for ORN, one catalytic, the other regulatory. When present in high concentration ORN binds to the regulatory site causing a conformational change in the OTCase protein which unmasks a binding site to the

arginase protein. In the absence of arginine the binding between OTCase and arginase is weak and the reducing effect of the induced conformational change on OTCase catalytic activity is low. In the presence of arginine the binding is strong and the inhibition of OTCase catalytic activity is high. The binding of arginase to OTCase has no effect on the former enzyme's catalytic activity (Messenguy et al, 1971; Wiame, 1971).

A further level of control may be exerted through the observed compartmentation of arginine pathway metabolites (Wiemkin and Durr, 1974) and enzymes (Jauniaux et al, 1978). These observations are of particular interest given the previously demonstrated regulatory interactions between arginase and ornithine transcarbamylase mentioned above. In several yeasts (Urrestarazu et al, 1977) and in Neurospora crassa (Weiss and Davis, 1973; Cybis and Davis, 1975) arginase is found in the cytosol while OTCase is confined to the mitochondria (Fig.2); in Saccharomyces cerevisiae, however, both enzymes occur in the cytosol so permitting the regulatory interaction of the arginase with OTCase. There appears to be a correlation between this regulatory interaction and the kind of terminal oxidant used for growth; obligate aerobes do not show the OTCase-arginase interaction whereas preferential anaerobes do (Urrestarazu et al, 1977).

In S.cerevisiae all enzymes of the acetylated intermediate cycle, including ACKase, are found in the mitochondria whereas those converting ORN to ARG are cytosolic, as are the catabolic enzymes OTCase and arginase (Jauniaux et al, 1978).

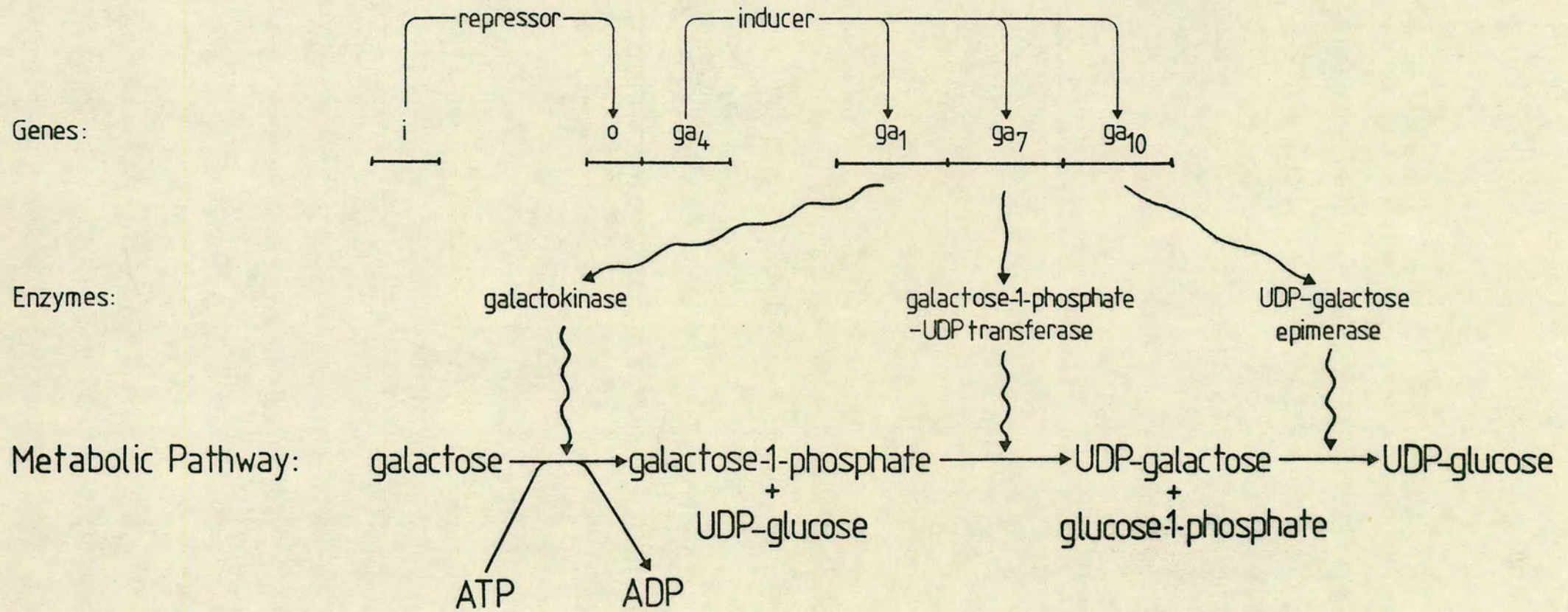
The mitochondrial location of all the acetyl-cycle enzymes is of interest since, as in N.crassa, AGKase is subject to inhibition by arginine in vitro (De Deken, 1962). A second mitochondrial enzyme, acetylglutamate synthase, is also inhibitable by arginine in vitro (Jauniaux et al., 1978). Again, as in N.crassa, the main regulatory signal for the pathway is believed to be the arginine cytosolic pool and the experimental evidence supports the contention that a feed-back inhibition loop of arginine on at least AGKase activity is operative in vivo (Hilger, et al., 1973 and Jauniaux et al., 1978). It has therefore to be presumed that an arginine mitochondrial pool exists in S.cerevisiae (and Neurospora crassa?) and assumed that this pool size is a reflection of the cytosolic pool size (Jauniaux et al., 1978).

The cytosolic location of OTCase and carbamylphosphate synthase is the exception rather than the rule in eukaryotes. It is interesting to note that carbamylphosphate is not detectable in S.cerevisiae (Lacroute et al., 1965).

In Neurospora the mitochondrial location of the anabolic enzymes producing and utilising ORN together with the cytosolic location of the enzymes catabolising ornithine has been considered to be a powerful mechanism for minimising "potentially wasteful" cycling of endogenously synthesised ORN (see 1.3.2.1). In S.cerevisiae the OTCase-arginase interaction has been interpreted as an efficient mechanism for the prevention of the "potentially wasteful" cycling of ORN presumed to otherwise follow from the colocation in the cytoplasm of these two enzymes (Jauniaux et al., 1978).

As in Neurospora (see 1.3.2.2.2) (and also Candida utilis

Figure 78: Galactose Utilization in *Saccharomyces cerevisiae*.



(Wienkin and Nurse, 1973)), the vast majority of the arginine and ornithine pools in S.cerevisiae are sequestered from their cytosolic pools in a membrane bound subcellular organelle, the vacuole (Wienkin and Durr, 1974). The conclusions drawn from analysis of Neurospora as to the regulatory effect of metabolic compartmentation are probably applicable to S.cerevisiae (Davis, 1975).

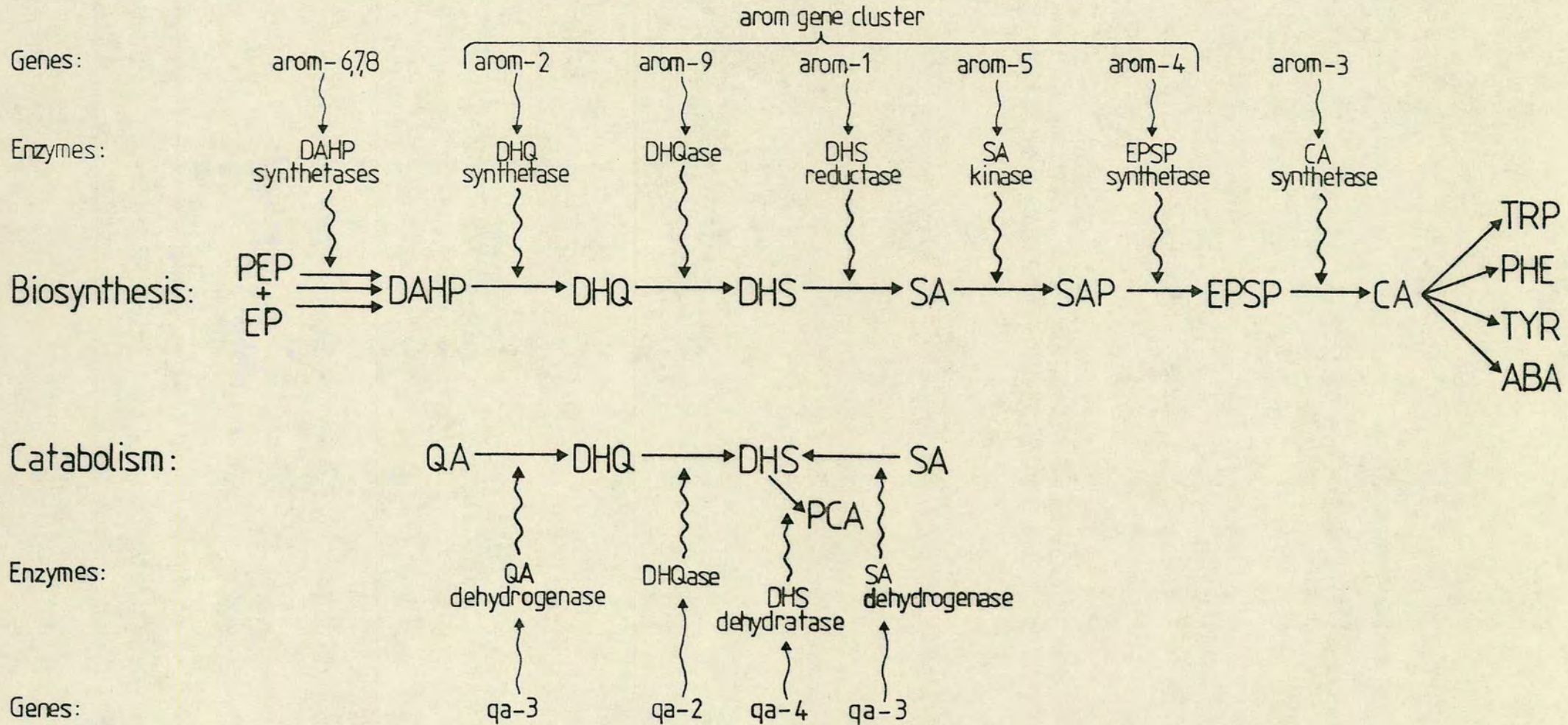
In summary, the overall similarities in the structure, organisation and regulation of arginine metabolism in the two relatively closely related eukaryotes, Neurospora crassa and Saccharomyces cerevisiae, are perhaps not surprising. The specific dissimilarities are however particularly interesting in that they illustrate the scope for variation in the solution to a problem of metabolic control. The studies of Hilger et al (1973) in Saccharomyces and Barthelmess et al (1974) in Neurospora, for example, show that although specific regulatory phenomena may differ in detail between the two systems the control of flux is a consequence of the same universal systemic properties.

(b) Galactose utilization in Saccharomyces cerevisiae

In the introduction to this thesis the lack of success in the search for the "operon" in eukaryotes was commented upon. While not strictly conforming to the lactose-utilisation system of E.coli K12, the genetic system for galactose utilization in Saccharomyces embodies several features of the bacterial operon (see Figure 78).

The gal region in S.cerevisiae consists of three, probably distinct but tightly linked, loci (ga₁, ga₂ and ga₁₀) (Douglas and Pelroy, 1963); expression of this region of the genome is

Figure 79: Aromatic amino acid biosynthesis and quinate-shikimate catabolism in *Neurospora crassa*.



Legend to Figure 79:

PEP	Phosphoenolpyruvate
EP	Erythrose-4-phosphate
DAHP	Deoxyheptulosonic acid phosphate
DHQ	Dehydroquinic acid
DHS	Dehydroshikimic acid
SA	Shikimic acid
SAP	Shikimic acid phosphate
EPSP	Enolpyruvylshikimic acid phosphate
CA	Chorismic acid
TYR	Tyrosine
TRP	Tryptophan
PHE	Phenylalanine
ABA	p-aminobenzoic acid
QA	Quinic acid
PCA	Protocatechuic acid

positively regulated by the product of the unlinked ga₁ locus (Douglas and Hawthorne, 1964). The ga₁ locus in turn is negatively controlled by the product of another unlinked locus, i (Douglas and Pelroy, 1963); the i gene product is presumably a repressor protein which binds to the operator site (identified by mutation) closely linked to ga₁ (Douglas and Hawthorne, 1966).

(c) Aromatic aminoacid synthesis and quinate-shikimate degradation in *Neurospora crassa*.

These metabolic pathways, their intermediates, enzymes and genetic determinants, are illustrated in Figure 79. The essential features are:

(a) a multienzyme aggregate coded for by a gene cluster (arom); the multienzyme aggregate "minimizes the escape of intermediates" from the biosynthetic pathway into catabolic pathways,

(b) a degradative pathway from quinic and shikimic acid; seen only when synthesis of the constituent enzymes of the pathway is induced by the accumulation of a sufficiently high concentration of quinic acid or one of the intermediates of the degradative pathway.

Although the anabolic and catabolic pathways have two intermediates in common (dehydroshikimate and dehydroquininate, see Figure 79) the above features effectively prevent competition between the two pathways for common intermediates.

The arom mutants affect the biosynthesis of chorismate, the common precursor of the aromatic aminoacids tyrosine, phenylalanine, tryptophan and p-aminobenzoic acid. Seven enzyme-catalysed steps are involved in the synthesis of chorismate from PEP and EP. The first step is catalysed by the product of three different genes

(arom-6, 7, 8) each gene coding for a different allosteric enzyme; the activity of the iso-enzymes is regulated by the different aromatic end-products subsequently formed from chorismate (Halsall and Doy, 1969). The last enzyme involved in the synthesis of chorismate, chorismate synthetase, is deficient in arom-3 mutants. The structural gene(s) coding for the other five enzymes involved in chorismate synthesis all map in a tight cluster a few map units away from arom-3 on linkage group II, (Giles et al, 1967). This gene cluster (arom-1, 2, 4, 5 and 9) was thought originally to be a good candidate for a fungal operon; the tight linkage, results of complementation studies, and existence of pleiotropic mutants were taken as evidence. However, neither regulator nor operator type mutants have yet been isolated. Although mutations affecting each of the individual enzymes are known, the five gene products are dependent upon each other for activity. All five enzymes activities are associated with a single protein or with an aggregate of several proteins (Burgoyne et al, 1969). From the genetic data the five gene-five polypeptide hypothesis was favoured but recent work (Lumsden and Coggins, 1977; Gaertner and Cole, 1977) suggests that the arom-gene cluster codes for a single polypeptide with five enzyme activities. If correct, this would explain complementation data hard to reconcile with the five gene-five polypeptide hypothesis.

Of considerable importance for an understanding of control of flux through alternative pathways is the observation that the enzyme aggregate effectively sequesters intermediates in the biosynthetic pathway to chorismate. Intermediates derived from DAHP are used more effectively than the same intermediates added directly to the isolated enzyme aggregate (Gaertner et al, 1970).

The quinate-shikimate degradative pathway is specified by a gene cluster coding for three inducible, non-aggregated enzymes (Chaleff, 1974a,b). These three enzyme activities are strictly coordinately expressed, implying control of expression at the level of transcription with the possible existence of a polycistronic messenger and an operon-type system for regulating flux through the degradative pathway (Chaleff, 1974a & b).

A regulatory gene, qua-1, has been defined by the induction and selection of negative and constitutive mutations having pleiotropic effects on the synthesis of the catabolic enzymes. This gene (mapping at one end of the gene cluster) codes for a mutated protein which acts positively to turn on the transcription of the structural genes, qua-2, qua-3 and qua-4 (Valone *et al.*, 1971 and Partridge *et al.*, 1972). Genetic analysis suggests the existence of contiguous but non-overlapping DNA segments coding for the inducer and the DNA-binding regions of the regulatory protein (Case and Giles, 1975).

Synthesis of the catabolic enzymes is induced by (in addition to quinate itself) the intermediates dehydroshikimate and dehydroquininate, intermediates which are common to the anabolic and degradative pathways (Chaleff, 1974b) (Figure 79). That the catabolic enzymes are present at only very low activities in uninduced wild-type strains of Neurospora crassa implies that the anabolic enzyme "aggregate" sequesters enough of its intermediates to keep the concentrations of the free solutes sufficiently low to prevent induction of the synthesis of the enzymes of the catabolic pathway. This sequestration of

substrates may also mean that the enzyme concentration approximates to the free substrate concentration - the consequence of which has already been discussed previously with respect to CAP channelling in Neurospora.

(d) Aspects of Ammonium, Nitrate and Nitrite Metabolism in Fungi.

Investigation of the regulation of nitrate reduction in A.nidulans, initiated by Pateman and Cove, has revealed a system of considerable interest which appears to increase in complexity with each new publication. To do justice to this topic would require a complete thesis but an outline account will be useful.

Nitrate is reduced to ammonium by the sequential action of nitrate reductase, nitrite reductase and hydroxylamine reductase. Synthesis of each of these enzymes is induced by nitrate and repressed by ammonium. Expression of the nia-D (nitrate reductase) and nia-A (nitrite reductase) genes is regulated by the product of the nir-A (synonymous with nii-B) locus (Pateman and Cove, 1967; Cove and Pateman, 1969). The product was thought to be an inducer which is stabilised by nitrate reductase when the latter is complexed with nitrate but which acts as a repressor in the presence of ammonia. Further evidence which showed that the level of nitrite and nitrate reductase activity in nir-A^C/nir-A⁺ heterokaryons is intermediate between that in the two homokaryons is however consistent with the hypothesis that nir-A gene product acts only as an inducer but is formed in limiting amounts (Cove, 1969). That enzymatically active nitrate reductase is required for induction was deduced from the analysis of another type of mutation symbolised onx

(Cove and Pateman, 1963; Pateman et al., 1964). The onx genes, mapping at five different loci, are all required for the synthesis of a molybdenum cofactor of both nitrate reductase and xanthine reductase activity. The nia-D gene codes for a protein with cytochrome c reductase activity and, in conjunction with functional onx gene products, nitrate reductase activity (Pateman et al., 1964). When inactive nitrate reductase is formed (in either onx or nia-D mutants) nitrite reductase and (in onx mutants) cytochrome c reductase are produced constitutively in the absence of nitrate although they are still repressible by ammonium (Pateman et al., 1964).

An additional level of control of protein synthesis is imposed by the mea gene product (Arst and Cove, 1969). Mutations designated mea^R (selected as resistant to the growth inhibitory ammonium analogue methylamine) contain a nitrate reductase, synthesis of which is not repressed by ammonium; the same is true in these mutants for other enzymes which have ammonium as an end-product.

As suggested above, many catabolic enzymes are sensitive to Nitrogen (or Ammonia) repression. In S.cerevisiae the primary route for ammonium assimilation is through NADP-linked glutamate dehydrogenase (NADP-GDHase) and, like nitrate reductase, this protein has both a catalytic and regulatory function; structural gene mutants (gdh-A) (Grenson et al., 1974) lead to a loss of ammonia repressibility of (i) the synthesis of enzymes involved in the catabolism of N-containing compounds (Grenson, et al., 1974; Dubois et al., 1973 and 1974) and (ii) the assimilation/translocation of aminoacids (Grenson and Hou, 1972), (see preceding

section for ammonium repression of arginase synthesis in S.cerevisiae). The data suggests that when NADP-GDHase is complexed with both its substrates (ammonium and α -ketoglutarate) it acts as a repressor or negative regulator of the synthesis of enzymes in ammonia-yielding pathways.

A similar regulatory role for NADP-GDHase in A.nidulans has been proposed on the basis that, as in Saccharomyces, structural gene mutations (gdh A) lead to the loss of ammonia repressibility of many catabolic enzymes (Pateman et al., 1973 and Kinghorn and Pateman, 1973). Ammonia-repressibility is also affected by the product of a second gene, areA ; since these mutations are epistatic to gdh A mutations it has been proposed that the areA gene product mediates the action of the NADP-GDHase ammonia regulatory complex (Arst and Cove, 1973).

NADP-GDHase is by no means the only enzyme whose activity is regulated by the areA gene product. Indeed, the areA gene product appears to define a general level of control, acting positively on the expression of all known ammonium repressible enzymes and permeases (Arst and Cove, 1973). Furthermore, there appears to be an absolute requirement for a functional areA gene product (either wild-type or mutant, with altered specificity) for the expression of each of the respective structural genes (Arst and Scazzocchio, 1975). The several different areA mutants studied exhibit a varied spectrum of phenotypes with respect to the expression or otherwise of these activities (Arst and Cove, 1973), an observation which suggests that the (presumed) receptor sites for the areA gene product, i.e. "initiator" genes, may be different

for different (sets of) structural genes (Arst and Scazzochio, 1975). The absence of structural gene expression in the absence of functional areA gene product provides a potential means for identifying such "initiator" regions by selecting for cis-acting mutations which permit the expression of the adjacent structural gene through facilitating the binding of the mutant areA gene product (Arst and Scazzochio, 1975). However, this approach is not generally applicable since in Aspergillus the structural genes for sequentially acting, ammonium repressible enzymes are frequently unlinked and therefore reversion would require the highly unlikely, simultaneous mutation of several different cis-acting regulatory loci. In the particular case, though, the method has met with success.

Arst and Scazzochio (1975) successfully exploited the particular properties of one mutant, areA-102, to select for initiator mutations. The areA-102 mutant, although affecting additional activities, is most notably characterised by the almost total lack of uric acid or xanthine transport activity, while the catabolism of these compounds once inside the cell is quite unaffected (Arst and Cove, 1973). They therefore selected, after NTG mutagenesis the areA102 strain, for conidiospores able to grow on medium with uric acid as the sole nitrogen source. All but one of the revertants were presumed by their phenotypes to be second-site areA mutations but the last revertant, designated uap-100, was found to have exclusively regained the ability to utilise uric acid (and xanthine) but otherwise retaining the characteristics of the are102 strain. The uap100 mutation was found to result in strongly constitutive uric acid-~~xanthine~~ permease activity being some 16-

fold higher than wildtype in the absence of inducer and having a maximal level of induction 2.5 fold higher than wild-type. A similar "up-promoter" effect has been observed for many initiator constitutive mutations in E.coli.

The presumptive structural gene for the permease, uapA, was shown to be very tightly linked to the uap-100 mutation. Furthermore the expression of the former was only effected by the latter when located in the cis-configuration. The genetic and biochemical data are most consistent with the hypothesis that the uap-100 mutation defines a cis-acting control region adjacent to the permease structural gene and that the uap-100 suppression of the areA102 mutation is due to the former accommodating the binding of the mutationally altered product of the latter (Scazzochio and Sealy-Lewis, 1978).

As well as being subject to areA mediated repression by ammonium, the induction of uricacid-xan thine permease (by its substrate, uricacid, or the gratuitous inducer 2-thiouricacid) is mediated by a second positive control gene, uaY (Scazzochio et al, 1973). As with the areA gene, a functional uaY gene product had been shown to be essential for uapA expression, a requirement not alleviated by the uap100 mutation (Scazzochio and Arst, 1978). Thus, the evidence strongly suggests that, as for the areA gene product, there must be a receptor site for uaY gene product adjacent to the uapA structural gene.

The effect of the uap100 mutation is at least three-fold. Firstly, it permits the binding of the mutant areA-102 gene product, secondly, it permits the binding of the uaY gene product in the

absence of coinducer (thus leading to constitutively induced enzyme synthesis) and, thirdly, it affects the (presumed) promoter region such that the maximal level of induction is 2.5-fold higher than in wild-type. The implication is that at least a functional overlap must exist between the receptor sites for the uaY and areA gene products respectively and the promoter site.

The nature of the uaY gene product has been characterized further. The activity of the uaY shows nuclear limitation (Scazzochio and Darlington, 1967; Scazzochio et al., 1973); uapA gene expression is only observed when a functional uaY gene is present in the same nucleus, either in the cis-configuration in heterokaryons or in both the cis- and trans-configurations in diploids (Scazzochio and Arst, 1978). Recently the protein product of the uaY gene has been tentatively identified (Phillippides and Scazzochio, 1978), the first time that this has been achieved for a formally defined regulatory gene in a eukaryote. The preliminary characterization of this protein suggests that it may exist in alternative conformations, a property consistent with its proposed regulatory role (Phillippides and Scazzochio, 1978; Scazzochio and Arst, 1978).

Finally, mention must be made of the work of Arst and MacDonald (1975) on the regulation of proline catabolism in Aspergillus nidulans. They isolated mutants deficient in the structural components of the system, namely proline transport (prnB⁻) and the two enzymes proline oxidase and pyrroline-5-carboxylate dehydrogenase defined by mutations at a single locus, prnA⁻. Mutations at these two loci, all recessive, complemented one

another in both heterokaryons and diploids, and were shown to be tightly linked to each other, defining a functionally related gene cluster (Arst and MacDonald, 1975).

The two catabolic enzymes (but not proline transport) are ammonium repressible and thus the expression of the prnA (but not the prnB locus) is under areA control (Arst and MacDonald, 1975). Mutations, designated prn^d, can partially suppress the effect of areA mutations, and uniquely permit the utilisation of proline as a nitrogen source. This ability is probably affected through the relief of carbon catabolite repression since proline transport is derepressed despite being outwith areA control (Arst and MacDonald, 1975).

The most dramatic finding of this study, however, was that not only are the two structural genes, prnA and prnB, tightly linked but that the regulatory mutation, prn^d, maps between the two. All the genetic evidence from meiotic analysis, epistatic relationships and complementation tests consistently identified the prn^d mutation as being outwith but between the prnB and prnA loci. That the regulatory function of prn^d was active only in the cis-configuration, together with the other aspects of the systems behaviour was consistent with the prn^d mutation defining an operator-type region for the negatively controlled carbon catabolite repression system (Arst and MacDonald, 1975). This was, in the words of the authors, "the first definitive demonstration of an operon-type structure (as opposed to simple tight linkage of functionally related genes) in an eukaryotic organism". The authors did speculate that the observed gene arrangement would

permit the co-ordinate control by carbon catabolite repression of both prnA and prnB with the independent control of prnA (but not prnB) by ammonium repression; however, such an arrangement is by no means prerequisite for such a variety of control to be mediated.

In summary, the control of general nitrogen metabolism is of paramount importance to cellular economy and survival and as such the complexities and hierarchies of regulatory mechanisms are of particular interest to the geneticist. More than a cursory excursion^{into} the ramifications of this topic, as illustrated by genetic analysis of the fungi, has been beyond the scope of this thesis. Nevertheless the preceding description illustrates again the importance of proteins with both catalytic and regulatory functions and in particular the potential of a single enzyme-substrate interaction to mediate control over an extensive area of metabolism.

The observation that different metabolic activities frequently occur at distinct but closely co-ordinated times within the cell cycle has lead to suggestions of still more general levels of genetic control and metabolic integration (Hartwell, 1974; Mitchison, 1971). An example of "general" control of particular relevance to the present work is the "cross-pathway" regulation of aminoacid biosynthesis in Neurospora crassa.

(e) Cross-pathway regulation of aminoacid biosynthesis in
Neurospora crassa

Carsiotis and Lacy (1965) reported that in his auxotrophs grown on limiting histidine the level of tryptophan synthetase activity, in addition to the derepression of histidine pathway anabolic enzymes, was elevated 2- to 3-fold over that observed in

wild-type minimally grown cultures. Under the same experimental conditions of limiting histidine the levels of four other tryptophan pathway enzymes were shown to be similarly derepressed (Carsiotis et al., 1970).

Derepression was not due to a lowered intracellular concentration ^{of tryptophan} nor could it be reversed by the exogenous supply of tryptophan (Carsiotis et al., 1970). Derepression was not due to the accumulation of some histidine pathway intermediate(s) since it was observed to occur in his-2 mutants which are blocked at the first step in the histidine biosynthetic pathway (Carsiotis et al., 1970). It was subsequently shown that under these starvation conditions the enzymes of the arginine biosynthetic pathway were also derepressed and that this was not reversed by arginine supplementation (Carsiotis et al., 1974). In a reciprocal manner a 2- to 3-fold derepression of the tryptophan, histidine and arginine biosynthetic enzymes is observed in the tryp auxotrophs grown on limiting tryptophan (Carsiotis and Jones, 1974).

On the basis of these observations and the similar effects consequent upon the inhibition of aminoacyl-tRNA synthetase activities (Turner and Matchett, 1968; Nazario, 1967) Lester (1971) proposed a polycorepressor model to account for cross-pathway regulation. The essence of the model is that the active polyrepressor for arginine, histidine and tryptophan biosynthesis requires the presence of the amino acyl-tRNAs formed by each pathway; an insufficiency in any one leads to the derepression of the enzymes of each of the pathways and can only be reversed by the subsequent addition of the originally limiting amino acyl tRNA. In fact

there is no direct evidence for the existence of a polycorepressor; the model is simply a formal explanation for the experimental observations. It must be assumed that, as in S.cerevisiae, there are at least two types of regulatory "circuit", one "general" to several different biosynthetic pathways (i.e. the "cross pathway" circuit) and another "specific" to particular pathways since there is evidence for derepression of enzymes in, for example, the arginine pathway without concomitant derepression of the histidine, lysine or tryptophan pathways (Barthelmess et al., 1974 and Dr. I.B. Barthelmess, personal communication).

APPENDIX II

AMINOACID TRANSPORT IN NEUROSPORA CRASSA

In Neurospora crassa several aminoacid transporting systems have been detected, their ontogeny studied, their partial composition examined and their kinetic characteristics defined (for a concise review, see Scarborough, 1973). At least five different aminoacid transporting systems have been identified in germinated conidia and mycelia. These systems have been defined kinetically by Pall (1969, 1970a, b, 1971) and his nomenclature will be used to identify ^{the} different systems.

System I. ("The neutral") is specific for most aromatic and neutral aminoacids, including L-tryptophan, L-phenylalanine, glycine, L-alanine and L-Leucine (Wiley and Matchett, 1966; Pall, 1969). The system also has moderate affinity for other aminoacids including L-histidine, L-serine and L-methionine (Pall, 1970a, b). System I activity is present maximally in young rapidly growing cultures and decreases markedly with age (e.g. in 3 day mycelial pads) (Pall, 1969). Mutants specifically deficient in this transport system (designated mtr) have been selected by their resistance to inhibition of growth in the presence of the L-tryptophan analogue, L-methyl-L-tryptophan (L-MT) (Lester, 1966; Stadler, 1966). Suppressors of the mtr mutations have also been selected. These suppressors may be (a) allele specific (due to reversion at the first mutational site or, if the first mutational event was a frameshift mutation, either a second intra-locus frameshift mutation or an unlinked frameshift suppressor mutation (Brink, 1972) or (b) non-specific (Stadler,

1965, 1967) due to a mutation in a different transport system (probably system II or III) resulting in an altered substrate specificity.

System II ("The general") (Pall, 1969) has a broad range of specificities for neutral, acidic and basic amino acids (Magill, et al, 1973). This activity is virtually absent from young rapidly growing cultures, only appearing in old (3 day) mycelial pads apparently starved of a carbon source. A "scavenger" role has been proposed for this transport system (Pall, 1969).

The neu^a mutant described by Magill et al (1972) is probably specifically deficient in System II activity.

System III ("The basic") (Pall, 1970a,b) is specific for the transport of the basic amino acids L-arginine and L-lysine; L-ornithine, L-canavanine and, to a lesser extent, L-histidine are also transported (Pall, 1970a, b; Bauerle and Garner, 1964).

System III activity is maximal in rapidly growing cells and decreases with the age of the culture and its synthesis appears to be regulated in parallel with gross protein synthesis (Pall, 1970a). A mutant, designated bat, specifically deficient in System III activity, has been selected (Thwaites, 1967; Thwaites and Pendyala, 1968, 1969).

System IV ("The acidic") (Pall, 1970b) is specific for the acidic amino acids L-cyst^eic acid, L- and D-aspartic acid and L-glutamic acid. There is little System IV activity in germinated conidia or in rapidly growing cells. A "scavenger" function has been proposed for this transport system since maximal activity is seen under conditions of carbon, sulphur or nitrogen starvation in ageing mycelial pads. System IV is, however, distinct from System II;

the latter has a much lower affinity for acidic aminoacids.

System V (Pall, 1971) is specific for L-methionine and L-ethionine. It is again thought to be a "scavenger" system since its activity is only observable in sulphur-starved mycelial pads (Pall, 1971). The activity of this system is absent in cys-3 mutants (Pall, 1971), but this is not thought to be the structural gene for a component of the transport system per se. It seems rather that the wild-type gene specifies a regulatory product; in the mutant, several enzyme activities normally present under sulphur-starvation conditions are also absent (Marzluf and Metzenberg, 1968).

Mutants affected in the transport of aminoacids are readily selected by the ability to confer resistance to the growth inhibitory effects of aminoacid analogues. Mutants deficient in specific transport system activities have been described. Mutants with more general permeation effects include the mutants un-t (Kappy and Metzenberg, 1965) and nap (Jacobson and Metzenberg, 1968) which are deficient in acidic (but not basic) aminoacid transport systems as well as neutral aminoacid transport systems. The temperature sensitive lethal un-t mutant may be a generalised membrane deficiency since potassium transport is also impaired and protoplasts are osmotically fragile (Kappy and Metzenberg, 1967).

Conidial transport systems have been investigated by DeBusk and co-workers; they have similar properties to those already summarised above, as described by Pall, for germinated conidia and growing mycelial cultures. The absolute rates of uptake of aminoacids in ungerminated conidia are in general considerably lower than in actively growing cells (Scarborough, 1973).

Competitive uptake studies have suggested the presence of a variety of aminoacid transport systems in ungerminated conidia (Roess and DeBusk, 1965). Selection for resistance to inhibition of growth by aminoacid analogues has permitted the selection of mutants (PnB) deficient in the transport of basic aminoacids (Roess and DeBusk, 1968) or (PnN) deficient in the transport of aromatic and neutral aminoacids (Wolfenbarger and DeBusk, 1971a). Transport studies with the double mutants (PnNB) revealed a general system for the uptake of a wide range of aminoacids (Wolfenbarger and DeBusk, 1971b). A mutant (PnG) deficient in this last uptake system has been isolated (Rao et al., 1975; Rao and DeBusk, 1975). There appears to be no system specific for the transport of acidic aminoacids in ungerminated conidia; they are apparently transported both by the neutral aminoacid system (with relatively high affinity but low transport capacity) and by the general aminoacid transport system (with relatively low affinity but high transport capacity) (Wolfenburger and DeBusk, 1972).

Regulation of aminoacid transport systems.

It is clear from these descriptions of the various transport systems that their activities change with the development of the organism; that is, their activities are modulated, presumably as part of the co-ordinated process of development. In addition, there may be a more immediate response to changes in the external environment. Railey and Kinsey (1976) made a detailed study of the ontogeny of aminoacid uptake activities in Neurospora.

During germination and growth of Neurospora conidia, System I and System II activities increased, while System III activity

decreased, with the onset of germination. System I showed two definite peaks of activity during logarithmic growth; the first, at about six hours, correlated with the completion of germ tube formation in conidia, the second, at about twelve hours, with the completion of primary hyphal branching. That both peaks of activity had the same K_m for L-phenylalanine and both peaks were abolished in mt mutants suggested that the same "binding protein/permease" species was responsible for System I activity throughout the life cycle.

System II activity increased continually up to twelve hours growth after which it declined rapidly. In fact the activity of this system decreased almost in parallel with the decrease in ammonium ion concentration in the medium, an observation hard to reconcile with the proposed "scavenger" role for System II proposed by Pall (1969).

System III activity was high in ungerminated conidia but decreased rapidly with the onset of germination. A constant specific activity was maintained during log phase growth up until 12 hours after which, like System II activity, it declined to a very low level.

The competitive uptake studies and analyses of aminoacid analogue-resistant strains suggest that the different aminoacid transport systems share, and most probably compete for, common cellular components (Sanchez, et al., 1972). Regulation of the synthesis of components of the aminoacid transport system may be co-ordinated with regulation of the synthesis of intracellular enzymes involved in the metabolism of the transported aminoacid;

the analogy with the established situation in bacteria (Cohen and Monod, 1957; Jacob and Monod, 1961) is obvious. The possibility that the same holds true for Neurospora is suggested by a) the cys-3 phenotype, affecting both enzymes activity and the induction of System V activity (Marzluf and Metzenberg, 1968). and b) the regulation of System II (and possibly also System III) transport by ammonia (Tisdale and DeBusk, 1970; Railey and Kinsey, 1976). Transport activity is also regulated by the internal aminoacid pool. Wiley and Matchett (1968) observed a decrease in the rate of tryptophan uptake when the internal tryptophan pool concentration was high; exogenously and endogenously derived pools were equally effective. From the kinetics of inhibition and requirement for protein synthesis they assumed that the phenomenon was enzyme mediated. Pall (1971) coined the term transinhibition to describe the observation that preincubation (with subsequent removal from the medium) with a particular aminoacid reduced the rate of uptake of the same and other aminoacids. The higher the affinity of an aminoacid for a particular transport system the greater was its ability to transinhibit the uptake of aminoacids by that system (Pall and Kelly, 1971 and Pall, 1971). To explain this phenomenon Pall (1971) suggested that either (a) the protein(s) involved in transport had two binding sites - one to bind an aminoacid present in the medium prior to its transport into the cell and a second to which an internal aminoacid could bind and, in so doing, prevent the transport in of external aminoacids, or (b) a "carrier protein" with a single binding site was in equilibrium between a "free carrier" state (able to bind and

transport in external aminoacids) and "carrier aminoacid complex" state (formed with internal aminoacids) with the point of equilibrium determined by the concentration of internal aminoacid and its affinity for the "carrier protein" binding site.

A further regulatory phenomenon is Substrate Repression (Nell and DeBusk, 1974); uptake activity is repressed in the presence of aminoacid(s) in the medium and derepressed on its (their) removal. Although the extent of "repression" is related to the affinity of the aminoacid for the transport system the phenomenon is distinct from simple competitive inhibition or transinhibition in that it is dependent on new protein synthesis.

The structure of transport systems.

Precise information on the structural components of the transport systems is lacking but several observations suggest that glycoproteins are part of these systems. Wiley (1970) showed that osmotic shock treatment of conidia reduced tryptophan transport by 90% without appreciable loss of cell viability; tryptophan binding proteins were released into the medium during such treatment of conidia. Stuart and DeBusk (1971) showed that KO1 extraction of conidia reduced L-arginine transport and released two glycoproteins with relatively high affinity for L-arginine. Both of these proteins were absent from mutants (PmB) deficient in basic aminoacid transport; one of the glycoproteins is absent from mutants (PmN) of the neutral aminoacid transport system. Stuart and DeBusk (1973) have also shown that L-arginine transport is

enhanced by addition of these glycoproteins to a crude lipid film in vitro.

No difference was observable under the scanning electron microscope after water extraction of wild-type and ^{single} (PmN or PmB) or double (PmNB) mutant conidia. After KC1 extraction however, the double mutants (but not the single mutants or wild-type) had visibly disrupted cell membranes (Travis et al, 1972).

Summary

To sum up, several stereospecific systems for aminoacid transport have been identified in Neurospora crassa. Their activity is dependent upon the developmental stage of the organism, on the intracellular and on the extracellular milieu. Different systems may share or compete for common cellular components. The activities of the transport systems are dependent, at least in part, on glycoproteins. Genetic loci affecting the synthesis of aminoacid metabolising enzymes may also affect the synthesis of components of the aminoacid transport systems. There is, in principle, no reason why these transport systems should not be subject to allosteric regulation of their activity in the same way that certain intracellular enzyme activities are regulated; this is true whether the transport systems are themselves enzymes or enzymatically inactive "carriers" or "translocators". The activity of aminoacid transport systems are thus likely to be, and indeed appear to be, subject to induction/repression and inhibition/activation mechanisms. The transport systems described in this section have all been cell-surface transport systems (or assumed to be such). Similar systems have been described in isolated mitochondria

and isolated vesicles (Boller et al, 1975); they are likely to be subject to the same kinds of regulatory devices.

APPENDIX III

EXPERIMENTAL RESULTS

The complete experimental details and results of analyses performed are given in the following tables:

(Abbreviations used in addition to those in Figures 1 and 2 are shown at the foot of the relevant table).

TABLE 1

Experiment I: the germination effect of HIS on arg-12 strains

Strain	HIS:CIT ratio (mM)	Mycelial Harvest		Yield (mg)	Mycelial Aminoacid Pools			
		Time elapsed since (h:min)	Inocul ⁿ		(μmol. 100 mg ⁻¹ d.wt.)			
			HIS add ⁿ	d.wt.	ARG	CIT	ORN	HIS
	2:1	24:00	24:00	192	6.95	0.81	1.93	21.2
	2:1	24:00	24:00	169	5.16	0.49	2.31	21.0
	2.5:1	24:00	24:00	60	2.55	0.36	9.22	27.4
<u>arg-12 ure</u>	2.5:1	24:00	24:00	18	2.10	0.30	8.68	16.4
	3:1 to 7.5:1	24:00	24:00	ND	-	-	-	-
	3:1 to 7.5:1	42:00	42:00	ND	-	-	-	-
	2:1 to 7.5:1	42:00	42:00	ND	-	-	-	-
<u>arg-12 ota aga</u>	2:1 to 7.5:1	42:00	42:00	ND	-	-	-	-

Abbreviation used:- ND, not detectable.

TABLE 2

Experiment II: the post-germination effect of HIS on arg-12 ure

Strain:	<u>arg-12 ure</u>
Conidial inoculum ($\times 10^6$ per flask)	3.2
Initial CIT concentration (mM):	0.4
Concentration of HIS added (mM):	0.8 to 2.8

HIS:CIT ratio (mM)	Mycelial Harvest		Yield (mg) d.wt.	Mycelial Aminoacid Pools ($\mu\text{mol. } 100 \text{ mg}^{-1} \text{ d.wt.}$)			
	Time elapsed since (h:min)	HIS add ⁿ		ARG	CIT	ORN	HIS
	Inocul ⁿ						
2:1	22:55	14:55	128	6.01	0.59	0.31	22.9
2.5:1	23:00	15:00	128	5.09	0.75	0.88	24.3
3:1	23:05	15:05	72	2.01	0.28	3.48	19.7
3.5:1	23:10	15:10	39	0.72	0.19	13.3	17.5
4:1	25:00	17:00	32	0.42	0.32	20.9	14.1
4.5:1	25:05	17:05	23	0.31	0.24	18.9	9.18
5:1	30:25	22:25	22	0.24	0.19	16.5	5.19
5.5:1	30:30	22:30	18	0.13	0.15	23.2	4.61
6:1	30:40	22:40	16	0.15	0.25	17.0	3.76
6.5:1	30:50	22:50	8	0.18	0.11	20.2	3.24
7:1	31:10	23:10	4	0.20	0.36	19.2	2.69

TABLE 3

Experiment III: the reversibility of HIS-mediated growth inhibition

Strain:	<u>arg-12 ure</u>
Conidial inoculum ($\times 10^6$ per flask):	15
Initial CIT concentration (mM):	0.4
Concentration of HIS added (mM):	2.0
Concentration of CIT-2 added (mM):	2.0

Sample	Mycelial Harvest			Yield (mg) d.wt.
	Time elapsed since (h:min)			
	Inocul ⁿ	HIS add ⁿ	CIT-2 add ⁿ	
CH 1	17:15	10:00	NA	22
CH 2	17:50	10:35	NA	25
CH 3	19:00	11:45	NA	26
CH + C 1	19:30	12:15	0:15	24
CH + C 2	19:45	12:30	0:30	27
CH + C 3	20:15	13:00	1:00	27
CH + C 4	20:45	13:30	1:30	29
CH + C 5	21:15	14:00	2:00	27
CH + C 6	22:00	14:45	2:45	31
CH + C 7	22:45	15:30	3:30	37
CH + C 8	23:30	16:15	4:15	37
CH + C 9	24:30	17:15	5:15	53
CH + C 10	25:15	18:00	6:00	64
CH + C 11	25:50	18:35	6:35	73

Abbreviation used: NA, not applicable

TABLE 4

Experiment IV : the post-germination effect of HIS on arg-12 ota aga

Strain:		<u>arg-12 ota aga</u>	
Conidial inoculum ($\times 10^6$ per flask):		9	12
Initial CIT concentration (mM):		0.04	0.04
Concentration of CIT-2 added (mM):		0.36	0.36
Concentration of HIS added (mM):		0.8 to 1.2	1.4 to 3.0

HIS:CIT ratio (mM)	Mycelial Harvest		Yield (mg)	Mycelial Aminoacid Pools			
	Time elapsed since (h:min)	Time since (h:min)		(μmol. 100 mg ⁻¹ d.wt.)			
	Inocul ⁿ	HIS add ⁿ	d.wt.	ARG	CIT	ORN	HIS
2:1	21:10	10:55	65	9.08	0.53	0.45	24.2
2.5:1	21:15	11:00	84	8.87	0.78	1.77	26.1
3:1	21:20	11:05	84	4.27	0.45	5.42	25.2
3.5:1	21:25	11:10	108	3.28	0.38	7.77	26.0
4:1	21:30	11:15	112	2.64	0.47	9.89	22.8
4.5:1	21:35	11:20	90	1.10	0.32	12.7	21.1
5:1	21:45	11:30	86	1.18	0.38	15.3	22.4
5.5:1	21:50	11:35	85	0.52	0.38	19.9	17.5
6:1	21:55	11:40	80	0.46	0.27	18.9	19.2
6.5:1	22:00	11:45	79	0.35	0.26	22.0	17.9
7:1	22:05	11:50	63	0.38	0.40	21.9	15.5
7.5:1	22:10	11:55	50	0.36	0.32	21.3	17.6

/ CIT-2 added at same time as HIS

TABLE 5

Experiment V: the effect of PUT on growth inhibition

Strain:	<u>arg-12 ure</u>	<u>arg-12 ota aga</u>	
Conidial inoculum ($\times 10^6$ per flask):	10	4	6
Initial CIT concentration (mM):	0.04	0.04	0.04
Initial PUT concentration (mM), where added:	0.0125	0.0125	0.0125
Concentration of CIT-2 added (mM):	0.36	0.36	0.36
Concentration of HIS added (mM):	0.8 to 2.4	0.8	1.6 or 2.4

Strain	PUT + or -	HIS:CIT ratio (mM)	Mycelial Harvest		Mycelial Aminoacid Pools				
			Time elapsed since (h:min)	Yield (mg)	(μmol. 100 mg ⁻¹ d.wt.)				
			Inocul ⁿ	HIS add ⁿ	d.wt.	ARG	CIT	ORN	HIS
<u>arg-12 ure</u>	+	2:1	19:45	8:45	107				
	-	2:1	19:50	8:50	92	7.32	0.56	0.82	36.8
	+	4:1	20:05	9:05	69				
	-	4:1	20:10	9:10	62	1.40	0.11	18.2	36.1
	+	6:1	20:25	9:25	43				
	-	6:1	20:30	9:30	43	0.50	0.17	23.2	25.2
<u>arg-12 ota aga</u>	+	2:1	19:55	8:55	80				
	-	2:1	20:00	9:00	83	5.86	0.49	0.34	52.6
	+	4:1	20:15	9:15	94				
	-	4:1	20:20	9:20	87	0.86	0.52	3.82	33.4
	+	6:1	20:35	9:35	63				
	-	6:1	20:40	9:40	62	0.46	0.12	13.7	26.3

† CIT-2 added at same time as HIS

TABLE 6

The effect of the post-inoculation delay in HIS addition on the growth rate and the mycelial HIS pool

The effect is illustrated by way of the data shown below from all growth experiments where a 5:1 ratio of medium HIS:CIT was employed. Where applicable, the highest mycelial HIS pool attained is shown.

Experiment	Strain	Table	Post-inocul ⁿ delay (h:min)	Mycelial HIS pool ($\mu\text{mol.100 mg}^{-1}$ d.wt.)	Mycelial doubling time (h:min)	Growth rate constant (k) (h ⁻¹)
I	<u>arg-12 ure</u>	1	00:00	NA	∞	0
I	<u>arg-12 ota aga</u>	1	00:00	NA	∞	0
II	<u>arg-12 ure</u>	2	08:00	5.19	21 h *	0.033 *
IV	<u>arg-12 ota aga</u>	4	10:15	22.4	ND	ND
VIIa	<u>arg-12 ure</u>	9	12:00	37.5	4 h 36 min	0.165
VIa	<u>arg-12 ure</u>	7	13:00	43.5	3 h 45 min	0.185
Xb	<u>arg-12 ure</u>	15	15:00	32.3	3 h 5 min	0.225
Xa	<u>arg-12 ure</u>	14	17:00	45.1	2 h 15 min	0.308

* Estimated from harvest yield assuming a doubling time of 2 h 15 min between inoculation and HIS addition.

Abbreviation used: NA, not applicable; ND, not determinable.

TABLE 7

Experiment VIa : mycelial harvest yields and aminoacid pools in arg-12 ure

Strain:	<u>arg-12 ure</u>			
Condition :	(a)	(b)	(c)	(d)
Conidial inoculum ($\times 10^6$ per flask) :	6.6	6.6	13.2	13.2
Initial CIT concentration (mM):	0.5	0.5	0.5	0.5
Concentration of HIS added (mM):	1.5	2.0	2.25	2.5

Flask Number	Mycelial Harvest			Medium BUN (A ₅₂₀)	Mycelial Aminoacid Pools								Flask Number
	Time elapsed since (h:min)	Yield (mg)			(μmol. 100 mg ⁻¹ d.wt.)								
	Inocul ⁿ	HIS add ⁿ	d.wt.		ARG	CIT	ORN	GLU	PRO	GLY	LYS	HIS	
(a) 1	20:05	7:05	86	2.07	3.82	0.56	1.05	8.63	ND	1.20	0.58	39.0	(a) 1
(a) 2	21:45	8:45	138	1.98	2.31	0.44	2.28	8.80	0.85	1.26	0.61	37.6	(a) 2
(a) 3	22:55	9:55	175	1.81	3.51	0.49	1.35	10.04	0.72	1.84	0.57	42.9	(a) 3
(a) 4	23:55	10:55	265	1.63	3.54	0.54	1.05	8.73	0.45	1.28	0.50	34.8	(a) 4
(b) 1	19:10	6:10	75	2.12	2.82	0.41	2.26	9.26	0.66	1.50	0.53	43.9	(b) 1
(b) 2	20:30	7:30	96	2.06	1.24	0.30	4.99	9.26	0.95	1.48	0.87	36.0	(b) 2
(b) 3	22:20	9:20	132	1.85	1.75	0.33	3.11	10.1	1.05	1.97	0.72	39.2	(b) 3
(b) 4	23:35	10:35	188	1.81	2.22	0.35	2.84	9.15	1.19	1.60	0.67	38.0	(b) 4
(c) 1	19:20	6:20	125	2.06	2.71	0.42	1.78	8.42	0.63	1.14	0.54	41.1	(c) 1
(c) 2	20:35	7:35	170	1.94	2.18	0.41	2.65	9.93	1.50	2.01	0.81	44.7	(c) 2
(c) 3	22:05	9:05	232	1.76	2.06	0.30	2.29	9.31	1.15	2.02	0.68	41.3	(c) 3
(c) 4	23:20	10:20	288	ND	2.03	0.26	2.94	9.23	0.85	2.12	0.68	40.0	(c) 4
(d) 1	19:25	6:25	125	2.04	2.19	0.32	2.77	8.05	0.80	1.15	0.60	43.5	(d) 1
(d) 2	20:45	7:45	172	1.98	1.49	0.27	4.07	8.11	1.24	1.50	0.86	38.2	(d) 2
(d) 3	22:15	9:15	209	1.89	1.28	0.22	4.66	7.76	1.10	1.67	0.95	38.2	(d) 3
(d) 4	23:30	10:30	224	1.73	1.72	0.27	2.60	9.01	1.34	2.68	0.75	38.7	(d) 4

TABLE 8

Experiment VIb: mycelial harvest yields and amino acid pools in ure

Strain:				<u>ure</u>									
Condition:				(a)	(b)	(c)	(d)						
Conidial inoculum (x10 ⁶ per flask):				7.0	7.0	14.0	14.0						
Initial CIT concentration (mM):				0.5	0.5	0.5	0.0						
Concentration of HIS added (mM):				0.0	2.0	2.5	2.5						
Flask	Mycelial Harvest			Mycelial Aminoacid Pools								Flask	
Number	Time elapsed since	Yield		(μmol.100mg ⁻¹ d.wt.)								Number	
	(h:min)	(mg)											
	Inocul ⁿ	HIS	d.wt.	ARG	CIT	ORN	GLU	PRO	GLY	LYS	HIS		
		add ⁿ											
(a) 1	19:15	NA	61	15.7	19.4	0.62	11.0	0.18	3.12	2.14	0.75	(a) 1	
(a) 2	22:20	NA	153	13.3	22.0	0.54	10.8	0.33	6.71	1.34	1.12	(a) 2	
(b) 1	19:25	6:55	58	5.02	0.32	1.21	10.8	0.19	3.72	2.59	36.3	(b) 1	
(b) 2	22:15	9:45	133	6.24	0.24	2.16	11.7	0.34	7.43	5.76	33.3	(b) 2	
(c) 1	19:10	6:40	139	3.73	0.32	1.54	8.82	0.12	5.47	1.48	37.6	(c) 1	
(c) 2	22:05	9:35	299	5.45	0.23	1.18	10.8	0.23	4.80	4.34	30.3	(c) 2	
(d) 1	19:00	6:30	144	3.76	0.07	1.37	11.4	0.31	5.58	3.14	38.0	(d) 1	
(d) 2	22:00	9:30	267	4.35	0.04	1.19	11.4	0.36	7.43	5.78	35.0	(d) 2	

Abbreviation used: NA, not applicable.

TABLE 9

Experiment VIIa: mycelial harvest yields, aminoacid pools and enzyme specific activities in *arg-12 ure*

Strain:		<i>arg-12 ure</i>						
Condition:	..	(a)	(b)	(c)	(d)	(e)	(f)	(g)
Conidial inoculum ($\times 10^6$ per flask):		8.0	8.0	8.0	8.0	8.0	8.0	8.0
Initial CIT concentration (mM):		0.50	0.30	0.50	0.45	0.40	0.35	0.30
Concentration of HIS added (mM):		0.0	0.0	2.0	2.0	2.0	2.0	2.0

Flask Number	Mycelial Harvest		Yield (mg)	Medium CIT (mM)	Enzyme Specific Activity (nmol.mg ⁻¹ protein.min ⁻¹)			Mycelial Aminoacid Pools (μmol. 100mg ⁻¹ d.wt.)								Flask Number
	Time elapsed since Inocul ⁿ (h:min)	HIS d.wt. add. ⁿ			OTase	Arginase	AOGTase	ARG	CIT	ORN	GLU	PRO	GLY	LYS	HIS	
(a) 1	17:25	NA	62	0.33	41.9	146	810	18.8	19.8	0.16	8.34	0.36	1.30	0.15	0.23	(a) 1
(a) 2	21:15	NA	176	0.18	41.6	219	838	15.9	17.1	0.21	7.27	0.54	1.26	0.20	0.25	(a) 2
(b) 1	17:30	NA	62	0.33	40.3	242	760	17.9	15.8	0.15	8.49	0.42	1.34	0.17	0.19	(b) 1
(b) 2	20:45	NA	162	0.06	43.4	191	663	15.8	11.4	0.26	8.75	0.50	1.90	0.22	0.20	(b) 2
(c) 1	17:40	5:40	51	0.41	22.7	194	663	3.65	0.51	0.90	8.87	0.87	1.20	0.69	40.4	(c) 1
(c) 2	18:40	6:40	62	0.42	25.3	137	773	2.63	0.41	1.23	8.30	0.79	1.40	0.65	37.4	(c) 2
(c) 3	20:10	8:10	91	0.28	21.8	87	837	4.98	0.35	2.17	8.36	0.83	1.50	0.61	38.7	(c) 3
(c) 4	21:20	9:20	107	0.31	20.7	77	779	1.92	0.21	2.15	8.58	0.92	1.50	0.59	36.0	(c) 4
(d) 1	17:45	5:45	53	0.36	18.9	140	756	2.49	0.34	1.40	7.76	0.69	1.19	0.63	38.0	(d) 1
(d) 2	26:25	9:25	101	0.37	19.6	74	1025	1.17	0.21	3.08	7.07	0.91	1.55	0.77	29.7	(d) 2
(e) 1	17:50	5:50	53	0.36	19.5	101	763	1.78	0.26	2.21	8.05	1.21	1.68	0.66	37.5	(e) 1
(e) 2	18:45	6:45	60	0.36	15.3	73	895	1.16	0.18	2.71	6.58	1.14	1.71	0.73	31.5	(e) 2
(e) 3	20:15	8:15	76	0.35	17.4	58	1120	0.65	0.17	5.67	7.22	1.61	1.49	1.08	29.5	(e) 3
(e) 4	22:00	10:00	81	0.33	10.9	61	1459	0.35	0.12	12.6	7.29	1.05	2.00	1.06	17.7	(e) 4
(f) 1	18:00	6:00	49	0.33	19.3	74	941	1.07	0.28	3.87	8.65	2.28	1.56	1.05	36.4	(f) 1
(f) 2	18:50	6:50	56	0.29	17.8	70	2249	0.51	0.21	6.53	6.45	1.89	1.68	1.16	28.1	(f) 2
(f) 3	20:25	8:25	70	0.29	18.2	55	1259	0.34	0.11	7.92	5.32	1.50	1.60	1.19	20.8	(f) 3
(f) 4	22:05	10:05	68	0.30	17.0	51	1622	0.38	0.11	12.1	5.91	1.51	2.27	1.20	20.6	(f) 4
(g) 1	18:05	6:05	47	0.26	17.0	65	1210	0.51	0.16	5.89	6.03	1.84	1.60	1.17	27.0	(g) 1
(g) 2	18:55	6:55	52	0.22	15.0	99	1198	0.37	0.12	9.50	6.13	2.16	1.96	1.44	25.4	(g) 2
(g) 3	20:30	8:30	58	0.25	15.7	58	1367	0.35	0.10	11.6	6.03	1.56	2.14	1.46	21.8	(g) 3
(g) 4	22:10	10:10	58	0.27	20.4	54	1342	0.29	0.06	12.1	3.81	1.21	2.10	1.21	12.7	(g) 4

Abbreviation used: NA, not applicable.

TABLE 10

Experiment VIIa: the rate of medium CIT depletion

Medium samples were taken with time, as shown below, from the CIT-only grown cultures (a) 2 and (b) 2 (see Table 9) and the concentration of CIT remaining was determined using BUN reagents after chromatographic separation of CIT from urea (Section 2.2.81). The "harvest yields" corresponding to each medium sampling are extrapolated from the growth rate for cultures (a) 1 and 2 and (b) 1 and 2 shown in Figure 13. The rate of medium CIT depletion determined by this analysis is shown in Figure 17. The results of the analysis are as follows:

Medium Sample Time elapsed since (h:min)	"Harvest Yield" (mg)	Medium CIT (mM)	
inocul ⁿ	d.wt.	(a) 2	(b) 2
12:30	15	0.42	0.27
14:30	27	0.35	0.20
16:30	48	0.34	0.22
18:15	82	0.36	0.17
19:05	99	0.32	0.13
20:40	153	0.22	NA
20:45	162	NA	0.06
21:15	176	0.18	NA

Abbreviation used: NA, not applicable.

TABLE 11

Experiment VIIb: enzyme specific activities in ure

Strain:

Condition:	<u>ure</u>			
	M	C	CH	H
Conidial inoculum ($\times 10^6$ per flask):	7.0	7.0	7.0	7.0
Initial CIT concentration (mM):	0.0	0.5	0.5	0.0
Concentration of HIS added (mM):	0.0	0.0	2.0	2.0

Flask Number	Mycelial Harvest			Enzyme Specific Activity				Mycelial Aminoacid Pools								Flask Number			
	Time elapsed since (h:min)	Yield (mg)		(nmol.mg ⁻¹ protein.min ⁻¹)				(μmol.100mg ⁻¹ d.wt.)											
				Inocul ⁿ	HIS add ⁿ	d.wt.	OTAase	Arginase	AOGTase	ASA ase	ARG	CIT	ORN	GLU	PRO		GLY	LYS	HIS
M 1	21:25	NA	233	25.1	75	554	12.9	2.11	0.14	0.68	7.71	0.16	0.73	0.28	0.21	M 1			
M 2	21:50	NA	260	ND	72	830	8.7	2.23	0.13	0.58	8.27	ND	0.88	0.30	0.30	M 2			
M 3	23:00	NA	314	27.6	78	853	ND	2.11	0.17	0.54	8.11	0.31	1.08	0.36	0.26	M 3			
C 1	21:30	NA	179	80.6	534	903	10.4	17.3	18.3	0.25	8.23	0.46	2.4	0.18	0.27	C 1			
C 2	21:55	NA	197	88.0	588	ND	ND	15.3	15.5	0.24	7.01	0.27	1.62	0.23	0.26	C 2			
C 3	23:05	NA	288	79.3	581	653	ND	13.6	10.0	0.37	8.03	0.43	1.14	0.24	0.23	C 3			
CH 1	21:40	6:25	145	43.5	150	755	11.0	4.70	0.43	0.20	8.09	0.16	1.19	0.52	36.4	CH 1			
CH 2	22:05	6:50	155	50.0	107	ND	ND	4.49	0.43	0.16	9.17	ND	1.32	0.51	35.7	CH 2			
CH 3	23:15	8:00	149	40.6	136	758	ND	4.66	0.43	0.23	8.39	0.17	1.26	0.52	36.4	CH 3			
H 1	21:35	6:20	142	29.3	81	718	11.2	2.35	0.19	0.60	7.45	0.12	1.11	0.45	34.3	H 1			
H 2	22:00	6:45	151	33.7	95	ND	ND	2.60	0.24	0.62	8.42	ND	1.24	0.51	36.8	H 2			
H 3	23:10	7:55	200	32.8	90	836	ND	2.57	0.36	0.49	8.20	0.32	1.64	0.40	35.6	H 3			

Abbreviations used: NA, not applicable; ND, not determined.

TABLE 12

Experiment VIII : the stability of the steady state

Strain:	<u>arg-12 ure</u>
Conidial inoculum ($\times 10^6$ per flask):	18
Initial concentration of CIT (mM):	0.04
Concentration of 2nd CIT added (mM):	0.36
Concentration of HIS added (mM):	1.20

Flask Number	Mycelial Harvest			Enzyme Specific Activity				Mycelial Aminoacid Pools						Flask Number
	Time elapsed since (h:min)	Yield (mg)	d.wt.	(nmol.mg ⁻¹ protein.min ⁻¹)		(μmol.100 mg ⁻¹ d.wt.)								
	Inocul ⁿ	HIS add ⁿ		OTAase	Arginase	ARG	CIT	ORN	GLU	PRO	GLY	LYS	HIS	
CH 1	16:05	4:35	127	27.4	154	7.12	0.57	0.41	8.80	ND	1.05	0.49	34.9	CH 1
CH 2	17:05	5:35	152	25.8	175	5.12	0.36	0.38	7.11	ND	0.93	0.53	31.5	CH 2
CH 3	18:35	7:05	210	21.5	176	5.52	0.46	0.44	8.42	0.21	1.26	0.60	36.9	CH 3
CH 4	19:35	8:05	268	25.2	156	4.83	0.42	0.42	7.69	0.68	1.52	0.52	34.0	CH 4
CH 5	20:35	9:05	251	24.4	143	5.46	0.46	0.60	7.88	0.37	1.55	0.60	34.7	CH 5
CH 6	21:40	10:10	349	23.0	181	5.29	0.38	0.46	9.13	0.34	2.25	0.58	37.2	CH 6

Abbreviation used: ND, not determined

TABLE 13

Experiment IX: the effect of medium ARG and ORN on mycelial aminoacid pools, enzyme specific activities and fluxes in ure

Strain:			ure												
Condition:			M	A	AO										
Conidial inoculum (x 10 ⁶ per flask):			12.0	12.0	12.0										
Initial ARG concentration (mM):			0.0	1.0	1.0										
Initial ORN concentration (mM):			0.0	0.0	1.0										

Flask Number	Mycelial Harvest		Enzyme Specific Activity				Mycelial Aminoacid Pools								Flask Number
	Time elapsed since (h:min)	Yield (mg)	(nmol.mg ⁻¹ protein.min ⁻¹)				(μmol.100mg ⁻¹ d.wt.)								
			OTAase	Arginase	AOGTase	ASAase	ARG	CIT	ORN	GLU	PRO	GLY	LYS	HIS	
	Inocul ⁿ	d.wt.													
M 1	19:45	230	17.3	31	853	10.9	3.12	0.30	0.96	9.33	0.40	0.86	0.50	0.31	M 1
M 2	20:00	221	20.6	24	813	10.5	2.81	0.26	0.70	8.61	0.22	0.99	0.40	0.30	M 2
M 3	21:15	354	22.8	20	893	9.2	2.56	0.19	0.55	8.41	0.45	1.08	0.41	0.30	M 3
A 1	19:50	219	58.6	127	788	10.5	10.6	⋈	0.79	8.48	2.63	1.07	0.27	0.22	A 1
A 2	20:00	214	60.3	161	687	15.4	ND	ND	ND	ND	ND	ND	ND	ND	A 2
A 3	21:20	298	68.1	162	722	12.7	ND	ND	ND	ND	ND	ND	ND	ND	A 3
AO 1	19:55	225	59.4	124	729	10.1	10.1	⋈	1.70	9.80	3.21	1.04	0.29	0.24	AO 1
AO 2	20:05	248	59.7	99	666	11.0	10.1	⋈	1.72	9.36	3.11	1.04	0.24	0.24	AO 2
AO 3	21:25	327	66.0	123	698	10.9	7.89	⋈	1.69	8.77	2.75	1.08	0.19	0.19	AO 3

Abbreviations use: ND, not determined; ⋈, too small to quantify (less than 0.1 μmol.100 mg⁻¹ d.wt.)

TABLE 14

Experiment Xa: the post-HIS addition changes in mycelial aminoacid pools and enzyme specific activities in arg-12 ure

Strain: arg-12 ure
 Conidial inoculum ($\times 10^6$ per flask): 12.6
 Initial CIT concentration (mM): 0.4
 Concentration of HIS added (mM): 2.0

Flask Number	Mycelial Harvest			Enzyme Specific Activity			Mycelial Aminoacid Pools								Flask Number
	Time elapsed since (h:min)	Yield (mg)		(nmol.mg ⁻¹ protein.min ⁻¹)			(μmol.100mg ⁻¹ d.wt.)								
				Inocul ⁿ	HIS add ⁿ	d.wt.	OTAase	Arginase	AOGTase	ARG	CIT	ORN	GLU	PRO	
C 1	16:50	NA	37	79.3	372	865	18.7	16.9	0.33	7.57	0.37	0.70	0.19	0.24	C 1
C 2	22:20	NA	190	82.5	281	631	14.7	12.7	0.14	7.04	0.38	0.86	0.15	0.19	C 2
CH 1	17:10	0:10	40	72.4	400	837	17.2	12.5	0.26	6.97	0.33	ND	0.18	7.85	CH 1
CH 2	17:30	0:30	45	80.4	260	927	15.4	7.45	0.54	7.06	0.35	ND	0.15	16.7	CH 2
CH 3	18:00	1:00	51	66.1	224	818	15.9	6.44	0.39	7.40	0.39	ND	0.12	17.4	CH 3
CH 4	18:30	1:30	63	75.3	206	861	10.0	0.90	0.46	7.91	0.36	ND	0.23	24.6	CH 4
CH 5	19:00	2:00	76	65.9	177	937	9.4	0.83	0.42	10.5	0.36	ND	0.45	34.0	CH 5
CH 6	19:30	2:30	87	65.4	181	786	6.7	0.46	0.27	7.99	0.31	0.52	0.43	33.2	CH 6
CH 7	20:00	3:00	96	56.7	156	749	4.8	0.54	0.38	10.0	0.42	0.99	0.52	ND	CH 7
CH 8	20:30	3:30	112	58.5	143	761	4.6	0.28	0.38	6.83	0.33	0.63	0.42	38.8	CH 8
CH 9	21:00	4:00	114	53.8	92	765	4.7	0.38	0.76	5.36	0.68	1.20	0.62	ND	CH 9
CH 10	21:30	4:30	137	57.7	120	836	4.4	0.28	0.92	8.47	0.67	1.14	0.42	45.1	CH 10
CH 11	22:00	5:00	164	56.4	112	907	2.4	0.26	1.15	8.72	0.98	1.04	0.41	43.3	CH 11
CH 12	22:30	5:30	173	54.5	102	872	2.0	0.29	1.46	9.38	1.40	1.66	0.54	43.3	CH 12

Abbreviation used: NA, not applicable; ND, not determined.

TABLE 15

Experiment Xb: further post-HIS addition changes in mycelial aminoacid pools and enzyme specific activities in arg-12 ure

Strain:		<u>arg-12 ure</u>																
Conidial inoculum (x 10 ⁶ per flask):		20																
Initial CIT concentration (mM):		0.4																
Concentration of HIS added (mM):		2.0																
Flask Number	Mycelial Harvest			Yield (mg) d.wt.	Medium CIT (mM)	Enzyme Specific Activity (nmol.mg ⁻¹ protein.min ⁻¹)				Mycelial Aminoacid Pools (μmol.100mg ⁻¹ d.wt.)								Flask Number
	Time elapsed since (h:min)	Inocul ⁿ	HIS add ⁿ			OTAase	Arginase	AOGTase	ASAase	ARG	CIT	ORN	GLU	PRO	GLY	LYS	HIS	
C 1	14:40	NA	38	ND	58.9	347	902	18.6	20.2	15.2	0.44	6.55	0.49	0.91	0.20	0.31	C 1	
CH 1	15:10	0:10	51	0.271	56.1	380	952	16.4	17.3	12.4	0.28	6.83	ND	0.65	0.13	8.40	CH 1	
CH 2	15:30	0:30	54	0.301	55.9	306	711	14.9	15.2	9.74	0.42	7.92	ND	0.88	0.16	15.5	CH 2	
CH 3	16:00	1:00	64	ND	50.3	290	580	14.2	15.6	3.35	0.60	9.05	0.73	0.65	0.15	23.8	CH 3	
CH 4	16:30	1:30	67	0.277	46.8	241	627	15.1	10.4	1.25	0.64	7.11	0.73	0.62	0.15	22.9	CH 4	
CH 5	17:00	2:00	88	0.229	39.2	186	707	15.0	8.87	0.77	0.42	7.33	ND	0.73	0.34	25.3	CH 5	
CH 6	17:30	2:30	92	0.281	33.9	174	690	13.8	7.30	0.68	0.41	8.10	ND	0.90	0.43	25.1	CH 6	
CH 7	18:00	3:00	92	0.312	41.9	208	520	14.4	6.00	0.65	0.40	8.70	ND	1.08	0.44	29.6	CH 7	
CH 8	18:30	3:30	112	0.295	42.2	233	746	13.5	4.45	0.44	0.47	ND	0.42	1.00	0.38	28.4	CH 8	
CH 9	19:00	4:00	152	0.236	35.4	145	793	12.9	3.87	0.40	0.78	ND	0.69	1.33	0.39	31.1	CH 9	
CH 10	19:30	4:30	136	0.264	32.5	146	798	12.2	3.13	0.37	0.81	ND	0.59	1.20	0.38	31.8	CH 10	
CH 11	20:00	5:00	174	0.271	ND	138	811	13.4	2.92	0.38	1.23	ND	ND	1.23	0.41	32.3	CH 11	
CH 12	20:30	5:30	164	0.267	34.0	148	712	14.1	3.06	0.27	1.58	ND	1.01	1.23	0.43	26.3	CH 12	
CH 13	21:00	6:00	204	0.240	29.8	115	851	16.4	1.66	0.28	1.79	ND	1.01	1.41	0.42	28.2	CH 13	
CH 14	21:30	6:30	215	0.188	32.1	100	834	19.3	1.31	0.27	2.50	ND	1.20	1.77	0.50	25.3	CH 14	
CH 15	22:00	7:00	174	0.291	30.7	117	928	20.8	0.98	0.36	3.82	7.56	1.49	2.04	0.63	23.8	CH 15	
CH 16	22:30	7:30	266	0.226	32.9	37	1059	19.6	1.01	0.27	2.94	5.61	1.46	2.01	0.56	24.6	CH 16	
CH 17	23:00	8:00	319	0.199	31.5	96	1095	25.3	0.62	0.20	4.49	5.78	1.55	1.93	0.70	21.7	CH 17	
CH 18	23:30	8:30	322	0.205	32.1	ND	1378	30.5	0.83	0.30	4.21	5.96	1.99	2.80	0.79	23.8	CH 18	
CH 19	24:00	9:00	306	0.202	ND	50	1430	31.5	1.02	0.40	4.22	5.71	1.92	2.62	0.83	26.4	CH 19	

Abbreviations used: NA, not applicable; ND, not determined.

TABLE 16

Experiment XI: the "run up" condition in arg-12 ure

Strain: arg-12 ure
 Conidial inoculum ($\times 10^6$ per flask): 9.9
 Initial CIT concentration (mM): 0.4
 Concentration of HIS added (mM): 2.0
 Concentration of 2nd CIT added (mM): 2.0

Flask Number	Mycelial Harvest				Medium Urea (mM)	Enzyme Specific Activity				Mycelial Aminoacid Pools								Flask Number
	Time elapsed since (h:min)			Yield (mg)		(nmol.mg ⁻¹ protein.min ⁻¹)				(μmol.100mg ⁻¹ d.wt.)								
	Inocul ⁿ	HIS	CIT 2 add ⁿ			OTAase	Arginase	AOGTase	ASAase	ARG	CIT	ORN	GLU	PRO	GLY	LYS	HIS	
CH 1	20:00	7:20	NA	74	0.560	52.2	71	1151	19.5	0.86	0.33	4.09	6.21	1.47	1.42	0.91	21.2	CH 1
CH 2	20:30	7:50	NA	72	0.426	49.8	49	1538	21.3	0.88	0.33	4.39	6.58	1.36	1.50	0.96	24.2	CH 2
CH 3	21:30	8:50	NA	86	0.416	51.3	77	1274	21.7	0.90	0.46	5.83	7.12	1.46	1.76	1.13	23.4	CH 3
CH 4	22:30	9:50	NA	94	0.520	52.1	ND	1463	22.9	0.93	0.42	5.72	6.91	1.15	1.85	1.02	20.5	CH 4
CH + C1	20:50	8:10	0:10	85	0.610	50.5	68	1269	21.2	1.06	1.52	5.60	7.13	1.36	1.72	1.01	25.3	CH + C1
CH + C2	21:10	8:30	0:30	84	0.426	49.2	115	1134	18.3	2.40	2.10	3.60	5.89	0.65	1.33	0.74	21.2	CH + C2
CH + C3	21:40	9:00	1:00	90	0.600	52.8	102	1170	18.9	4.46	3.08	1.86	6.48	0.47	1.29	0.70	19.5	CH + C3
CH + C4	22:10	9:30	1:30	99	0.648	56.0	176	1243	19.6	6.17	3.97	1.70	7.30	0.35	1.18	0.71	22.6	CH + C4
CH + C5	22:40	10:00	2:00	111	1.32	54.1	126	1133	19.3	7.96	5.64	0.96	6.93	0.40	1.10	0.54	23.2	CH + C5
CH + C6	23:10	10:30	2:30	124	1.35	54.7	138	1243	18.5	10.4	7.68	0.68	7.27	0.41	1.37	0.53	21.5	CH + C6
CH + C7	23:50	11:10	3:10	127	ND	56.6	218	1067	17.6	14.5	8.03	0.67	8.92	ND	1.21	0.52	24.6	CH + C7
CH + C8	24:30	11:50	3:50	165	1.73	62.0	210	1075	18.2	13.9	7.61	0.58	7.57	0.39	1.08	0.43	21.6	CH + C8
CH + C9	25:30	12:50	4:50	198	1.16	70.3	309	1213	17.5	12.9	8.18	1.25	8.43	0.95	2.51	0.91	18.0	CH + C9

Abbreviations used: NA, not applicable; ND, not determined.

TABLE 17

Experiment XII: the 2:1 HIS:CIT "rundown" condition in arg-12 ota aga

Strain:		<u>arg-12 ota aga</u>												
Conidial inoculum (x 10 ⁶ per flask):		11.5												
Initial CIT concentration (mM):		0.04												
Concentration of 2nd CIT added (mM):		0.36												
Concentration of HIS added (mM):		0.80												
Flask Number	Mycelial Harvest			Medium		Mycelial Aminoacid Pools								Flask Number
	Time elapsed since (h:min)	Yield (mg)		CIT (mM)	HIS (mM)	(μmol.100mg ⁻¹ d.wt.)								
	Inocul ⁿ	HIS add ⁿ	d.wt.			ARG	CIT	ORN	GLU	PRO	GLY	LYS	HIS	
C 1,2,3	9:00	NA	13	ND	ND	20.1	2.68	1.09	8.04	0.40	1.02	0.55	0.55	C 1,2,3
CH 1,2	12:00	2:00	29	0.376	0.608	17.4	1.27	0.96	9.91	ND	0.87	0.60	ND	CH 1,2
CH 3,4	14:00	4:00	43	ND	ND	17.5	0.78	0.71	11.2	0.25	0.86	0.72	36.9	CH 3,4
CH 5,6	15:30	5:30	68	0.348	0.520	16.2	0.84	0.76	11.4	ND	1.27	0.86	37.9	CH 5,6
CH 7	17:30	7:30	50	0.320	0.565	14.8	0.85	1.09	11.2	ND	0.99	1.00	38.3	CH 7
CH 8	18:30	8:30	68	ND	ND	12.8	0.61	1.20	9.97	0.30	0.98	0.88	ND	CH 8
CH 9	19:30	9:30	84	0.297	0.523	13.7	0.78	0.60	9.97	0.39	1.01	0.91	33.6	CH 9
CH 10	20:30	10:30	105	ND	ND	13.7	0.67	0.67	9.66	0.30	1.23	0.89	35.7	CH 10
CH 11	21:30	11:30	121	0.291	0.519	13.8	0.99	0.75	10.4	ND	1.58	0.76	37.6	CH 11
CH 12	22:30	12:30	162	ND	ND	13.1	0.67	1.00	9.75	0.28	1.28	0.78	33.9	CH 12
CH 13	23:30	13:30	213	0.253	0.432	12.7	0.44	1.48	11.3	ND	1.22	1.03	33.3	CH 13
CH 14	24:30	14:30	250	ND	ND	12.7	0.65	1.66	9.85	0.32	1.57	0.99	31.7	CH 14
CH 15	25:30	15:30	333	0.160	0.393	10.1	0.63	1.34	10.4	ND	1.38	0.62	29.6	CH 15
CH 16	26:30	16:30	269	0.193	0.437	13.1	0.74	1.46	9.10	0.33	1.79	0.86	27.0	CH 16
CH 17	26:30	16:30	313	ND	ND	14.4	0.52	1.79	8.32	0.19	1.75	0.82	27.3	CH 17
CH 18	27:30	17:30	435	0.090	0.293	11.5	0.46	1.61	9.16	0.18	1.47	0.81	26.4	CH 18
CH 19	27:30	17:30	275	ND	ND	11.3	0.43	1.79	4.72	ND	1.52	1.20	24.1	CH 19

Abbreviations used: NA, not applicable; ND, not determined.

/ Added at same time as HIS

TABLE 18

Experiment XIII: the 3:1 HIS:CIT "rundown" condition in arg-12 ota aga

Strain :	<u>arg-12 ota aga</u>
Conidial inoculum ($\times 10^6$ per flask)	15
Initial CIT concentration (mM):	0.04
+ Concentration of 2nd CIT added (mM):	0.36
Concentration of HIS added (mM):	1.20

Flask Number	Mycelial Harvest			Mycelial Aminoacid Pools								Flask Number
	Time elapsed since		Yield (mg)	(μmol.100 mg ⁻¹ d.wt.)								
	(h:min)											
	Inocul ⁿ	HIS add ⁿ	d.wt.	ARG	CIT	ORN	GLU	PRO	GLY	LYS	HIS	
CH 1	15:00	4:30	49	12.7	0.53	1.31	11.3	0.73	0.97	0.68	41.3	CH 1
CH 2	17:00	5:30	63	10.9	0.42	1.29	9.48	0.23	0.79	0.80	37.4	CH 2
CH 3	17:50	6:20	76	10.7	0.48	1.63	9.39	0.24	0.89	0.93	42.3	CH 3
CH 4	19:30	8:00	98	9.68	0.51	2.18	ND	ND	1.14	0.99	44.1	CH 4
CH 5	20:30	9:00	112	8.71	0.41	2.41	9.39	0.14	1.04	0.56	37.3	CH 5
CH 6	21:35	10:05	158	9.08	0.48	2.23	9.29	0.22	1.33	0.88	37.5	CH 6

Abbreviation used: ND, not determined

+ Added at same time as HIS.

TABLE 19 Experiment XIV : the 4:1 HIS:CIT "run down" condition in arg-12 ota aga.

Strain:		<u>arg-12 ota aga</u>																
Conidial inoculum (x 10 ⁶ per flask):		2.4																
Initial CIT concentration (mM):		0.04																
Concentration of 2nd CIT added (mM):		0.36																
Concentration of 1st HIS added (mM):		0.80																
Concentration of 2nd HIS added (mM):		0.80																
Flask Number	Mycelial Harvest				Medium		Enzyme Specific Activity		Mycelial Aminoacid Pools									Flask Number
	Time elapsed since (h:min)		Yield (mg)		CIT (mM)	HIS (mM)	(nmol.mg ⁻¹ protein.min ⁻¹)		(μmol.100mg ⁻¹ d.wt.)									
	Inocul ⁿ	HIS 1 add ⁿ	HIS 2 add ⁿ	d.wt.					AOGTase	ASAase	ARG	CIT	ORN	GLU	PRO	GLY	LYS	
CH 1	20:30	9:30	NA	21	ND	ND	ND	ND	11.8	0.66	2.07	11.5	0.15	1.43	1.22	36.2	CH 1	
CH 2	21:30	10:30	NA	27	ND	ND	ND	ND	11.5	0.78	2.36	14.3	0.36	1.47	1.22	39.8	CH 2	
CH 3	22:30	11:30	NA	37	ND	ND	ND	ND	6.72	0.49	4.03	9.69	0.10	1.45	0.83	31.8	CH 3	
CH 4	23:30	12:30	NA	42	ND	ND	ND	ND	9.91	0.71	1.26	10.1	0.19	1.40	0.95	32.1	CH 4	
CH+H 1	22:00	11:00	1:00	33	0.368	1.39	884	10.2	7.77	0.36	2.22	9.12	ND	1.21	0.90	35.5	CH+H 1	
CH+H 2	23:00	12:00	2:00	43	0.358	1.28	922	9.8	4.72	0.42	5.35	9.77	0.15	1.53	0.89	34.5	CH+H 2	
CH+H 3	24:00	13:00	3:00	50	0.342	1.47	861	13.4	3.22	0.39	7.19	7.87	0.12	1.40	0.86	31.4	CH+H 3	
CH+H 4	25:00	14:00	4:00	65	0.361	1.42	1061	14.5	2.28	0.30	10.9	8.63	ND	1.68	0.85	30.0	CH+H 4	
CH+H 5	26:00	15:00	5:00	72	0.358	1.48	1088	16.7	1.56	0.33	12.0	7.19	0.23	1.83	0.82	24.4	CH+H 5	
CH+H 6	27:00	16:00	6:00	81	0.358	1.43	1181	15.9	1.49	0.30	17.0	7.58	0.27	1.96	0.81	25.7	CH+H 6	
CH+H 7	28:00	17:00	7:00	86	0.336	1.33	1145	14.7	2.16	0.39	14.0	6.97	0.26	1.89	0.97	27.6	CH+H 7	
CH+H 8	29:00	18:00	8:00	101	0.333	1.35	1274	20.5	1.63	0.35	17.6	7.03	ND	2.16	1.01	24.9	CH+H 8	
CH+H 9	30:00	19:00	9:00	115	0.347	1.39	1583	21.3	1.23	0.28	21.7	6.53	0.13	2.48	0.85	22.0	CH+H 9	
CH+H10	31:00	20:00	10:00	125	0.339	1.37	1625	22.1	1.11	0.23	20.6	6.11	ND	2.32	0.89	19.4	CH+H10	
CH+H11	32:00	21:00	11:00	126	0.311	1.32	1582	27.3	0.80	0.27	27.9	5.93	0.19	2.86	0.68	15.9	CH+H11	
CH+H12	33:00	22:00	12:00	147	0.265	1.22	ND	22.5	0.73	0.09	27.9	6.83	ND	2.93	0.71	16.3	CH+H12	

Abbreviations used: NA, applicable; ND, not determined

/ Added at same time as HIS-1

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Insert on p.253:

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